Origin, behaviour and control of enteric nervous system progenitor cells in Hirschsprung's disease

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

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Declaration

This thesis is the result of my own work. The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification

The research was carried out at the University of Liverpool

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Abstract

The enteric nervous system (ENS) arises mainly from cells exiting the vagal neural crest, entering the bowel and migrating caudally. A failure in this migratory process is thought to result in the clinical entity Hirschsprung's disease (HSCR), which is typically characterised by the absence of enteric ganglia in the colon and rectum (aganglionosis). Untreated HSCR may present with life-threatening bowel obstruction in the first few days of life. However, despite recent surgical advances children and adults still suffer significant life-long post-operative morbidity.

ENS progenitor cells (ENSPC) have been shown to persist in the postnatal bowel, thereby stimulating research into the potential of manipulating or transplanting these cells to improve long-term outcomes. Early results have been promising, ENSPC have been isolated from children with HSCR and clonally expanded in cultured neurospheres, after which they have been transplanted into aganglionic embryonic mouse gut ex-vivo and shown to restore a normal pattern of contractility. However, questions still remain about the functional potential and the safety implications of transplanting these cells back into humans. Therefore, this thesis is aimed at improving our understanding of the properties of these cells and the mechanisms controlling their behaviour when they are brought into culture and after subsequent transplantation.

The first part of this thesis focuses on the proliferation of ENSPC within the in-vitro environment of cultured mouse and human neurospheres. The main findings were that cells undergoing proliferation are predominantly found at the neurosphere periphery rather than being evenly distributed throughout the neurosphere. Subsequently, post-mitotic peripheral cells were found to be distributed throughout the neurosphere. Furthermore, using embryonic and postnatal mouse tissue it was demonstrated that the

proportion of cells undergoing of proliferation decreased with increasing maturity of the source tissue.

The second part of the thesis identifies the requirement for Notch signalling pathway in the self-renewal of undifferentiated human ENSPC. Inhibition of the Notch signalling pathway with both chemical inhibitors and siRNA knockdown resulted in decreased ENSPC proliferation together with increased neuronal differentiation. Manipulation of this pathway may therefore improve both the effectiveness and safety of any future cell-based therapy.

The next section of the thesis describes the development of an ex-vivo human colonic smooth muscle model to investigate the behaviour of ENSPC in an environment closer to that of the human colon. The chapter describes the development and maintenance of human colonic smooth muscle cells in long-term culture and demonstrates contractility, thus providing a model in which ENSPC behaviour can be investigated in a more physiologically relevant environment.

During the routine culture of human aganglionic smooth muscle preliminary observations were made consistent with the presence of cells that could give rise to neurospheres, despite the absence of an ENS in this region of the bowel. The final part of this thesis confirms the presence of ENSPC within the aganglionic bowel and characterises their behaviour. These progenitors are demonstrated to differentiate into mature ENS specific neuronal phenotypes, and importantly have the capability to restore a normal pattern of contractility in the embryonic aganglionic gut model.

Taken together the work in this thesis furthers our understanding of the control and behaviour of human ENSPC. Furthermore, demonstration of the existence of ENSPC in the bowel of children with HSCR raises important questions regarding both the aetiology HSCR and the potential to utilise the cells in future autologous cell-based therapies.

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Abbreviations

Abbreviations used within this thesis:

ANOVA	Analysis of variance
cGRP	Calcitonin gene related peptide
ChAT	Choline acetyl transferase
CIPO	Chronic intestinal pseudo-obstruction
CNS	Central nervous system
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DPC	Days post coitum
EDNRB	Endothelin receptor type-B
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
eM	Embryonic mouse
ENS	Enteric nervous system
ENSPC	Enteric nervous system stem cell
Et3	Endothelin 3
FBM	Fetal bovine media
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GDNF	Glial cell-line derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GRP	Gastrin related peptide
HCSMM	Human colonic smooth muscle model
H&E	Haematoxylin and eosin
HES	Hairy expresser of split
HESC	Human embryonic stem cells
Hey	Hairy expressor of split with YRPW motif
5-HT	5-hydroxytryptamine
HSM	Horse serum media

HSCR	Hirschsprung's disease
IBB	Immunofluorescence blocking Buffer
ICC	Interstitial cells of Cajal
LB	Lysogeny broth
NCC	Neural crest cell
NICD	Notch intracellular domain
nM	Neonatal mouse
NO	Nitric oxide
nNOS	Neuronal nitric oxide synthase
NPY	Neuropeptide Y
PACP	Pituitary adenyl-cyclase activating peptide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGP9.5	Protein gene product 9.5
RBP-Jκ	Recombination signal binding protein for immunoglobulin κ-J
RET	Rearranged during transfection
RT-PCR	Reverse transcriptase polymerase chain reaction
S100B	S100 Calcium binding protein B
SDC	Sodium deoxycholate
SFFV	Spleen focus forming vector
siRNA	silencing ribonucleic acid
SMA	Smooth muscle actin
SNM	Standard neurospheres media
TCM	Tissue Culture Media
TK	tachykinin
Tuj	Neuron-specific class III ß-tubulin
VIP	Vasoinhibitory peptide

Chapter 1: Introduction

Hirschsprung's disease (HSCR) is the commonest congenital neuropathy affecting the bowel and is caused by a failure of the enteric nervous system (ENS) to develop normally and colonise the distal bowel. The outcomes for HSCR have improved with advent of surgery to remove the affected bowel, however despite this both children and adults still experience significant life-long morbidity as a result of the disease. There has been growing interest over the last 10-15 years regarding the possibility of using ENS progenitors as a cell-based therapy to improve the current poor long-term outcomes. This chapter introduces the normal and abnormal development of the bowel and its nervous supply, it covers the known enteric neuropathies, specifically HSCR, summarises the current progress towards a clinical therapy and highlights the outstanding issues.

1.1. The Enteric Nervous System

The ENS is distributed throughout the length of the gastrointestinal tract providing intrinsic regulation of multiple processes including: muscle contraction, absorptions and immunity. It is network of neurons and glia with interconnecting ganglia organized into two plexi: the myenteric (Auerbach's) situated between the inner circular smooth muscle layer and the outer longitudinal muscle, and the submucosal plexus which lies between the inner smooth muscle and muscularis mucosa (Fig 1.1). Unlike the innervation of most organs, the ENS is capable of reflex activity independent of the brain and spinal cord. This is made possible by the presence of complete neuronal circuits, sensory neurons, afferent neurons, interneurons and efferent excitatory and inhibitory neurons (Furness 2005).

Figure 1.1. Organisation of the ENS

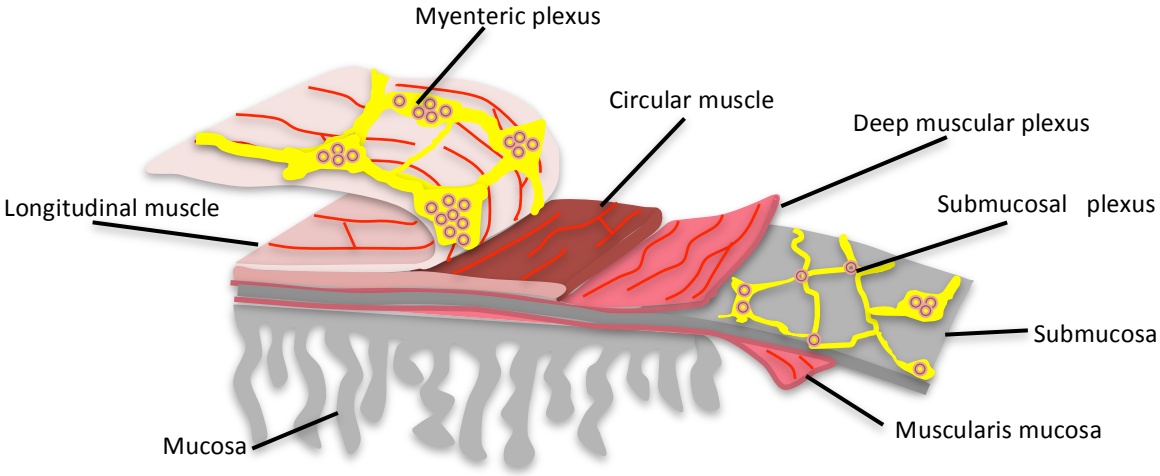


Figure 1.1 Organisation of the ENS. The myenteric plexus lies between the longitudinal and circular layers of muscle. The inner submucosal plexus lies below the circular muscle and above the muscularis mucosa. Adapted from (Furness 2005).

1.1.1. Function of the enteric nervous system

Comprising some 400-600 million neurons the ENS (Furness 2012) is involved in the regulation of a number of individual functions within the human gut, the primary one being to co-ordinate the widespread contraction seen within the bowel. This is achieved through the generation of two main types of propulsive wave (segmentation and peristaltic) promoting both the passage and mixing of intestinal contents as they progress proximally through the bowel. Other roles include the co-ordination of gastric acid secretion and absorption of nutrients, the regulation of blood flow within the bowel and local modulation of both the immune and endocrine systems (Furness 2005).

Given the wide range of functions it is of no surprise that the ENS is composed of more than 10 types neuron and greater than 15 neurotransmitters (Table 1.2). Including those that are unique to the ENS and others that integrate with both CNS and sympathetic / parasympathetic pathways. Our understanding of both the neurons and their associated transmitters is increasing but it remains to be seen whether the intricate arrangements and delicate balance between neuronal and hormonal control can be replicated with novel therapeutic modalities such as ENS progenitor transplantation.

Table 1.1. Common Neurotransmitters in the gut

Neuronal Type	Primary Transmitter	Associated Neurochemical markers	References
Enteric excitatory muscle motor neuron	ACh	TK, encephalin, cRET	(Brookes, *et al.* 1991, Holzer, *et al.* 1997, Grider 2003)
Enteric inhibitory muscle motor neuron	NO	VIP, TP, PACAP, carbon monoxide, opioids	(Fahrenkrug, *et al.* 1978, Costa, *et al.* 1992, K. M. Sanders, *et al.* 1992, Xue, *et al.* 2000)
Ascending interneuron	ACh	TK, ATP, cRET, encephalin	(Brookes, *et al.* 1991)
ChAT, nNOS descending interneuron	ATP, Ach	NO, VIP	(Young, *et al.* 1995, Brookes 2001)
ChAT, 5-HT descending interneuron	ACh	5-HT, ATP	(Furness, *et al.* 1982, Monro, *et al.* 2002, Gwynne, *et al.* 2007)
ChAT, Somatostatin descending interneuron	ACh	Somatostatin	(Portbury, *et al.* 1995, Gwynne, *et al* 2007)
Intrinsic sensory neuron	ACh, CGRP, TK	Calbindin, cRET	(Portbury, *et al* 1995, Grider 2003, Johnson, *et al.* 2004)
Interneurons supplying secretomotor neurons	Ach	ATP, 5-HT	(Surprenant 1984, Monro, *et al.* 2004)
Non-cholinergic secretomotor neuron	VIP	PACAP, NPY	(Cassuto, *et al.* 1981, Banks, *et al.* 2005)
Cholinergic secretomotor neuron	ACh	cRET	(Keast, *et al.* 1985, Young, *et al* 1995)
Motor neuron to gastrin cells	GRP, ACh	NPY	(Holst, *et al.* 1987, Weigert, *et al.* 1997)
Motor neurons to parietal cells	Ach	VIP	(Nilsson, *et al.* 1972, Feldman, *et al.* 1979)
Sympathetic neurons: motility inhibiting	Noradrenaline	NPY	(Finkleman 1930, Macrae, *et al.* 1986)
Sympathetic neurons: secretion inhibiting	Noradrenaline, ATP	-	(Dresel, *et al.* 1966, Costa, *et al.* 1984)
Sympathetic neurons: vasoconstrictor	Noradrenaline, ATP	NPY	(Dresel, *et al* 1966, Furness 1971, Furness, *et al.* 1983)
Intestinofugal neurons to sympathetic ganglia	ACh	VIP	(Crowcroft, *et al.* 1971, Dalsgaard, *et al.* 1983, Love, *et al.* 1987)

Table 1.1 Common neurotransmitters in the gut. Abbreviations: **Ach:** Acetylcholine, , **ATP**: adenosine triphosphate; CGRP: Calcitonin gene related peptide; GRP: Gastrin releasing peptide; NPY: Neuropeptide-Y; nNO: Neuronal nitric oxide PACAP: pituitary adenylyl-cyclase activating peptide; TK: Tachykinin; 5-HT: 5-hydroxytrypamine; **VIP**: Vasoinhibitory peptide. Adapted from Furness JB. The enteric nervous system and neurogastroenterology. *Nat Rev Gastroenterol.Hepatol 2012. **9**:286-294*

1.1.2. Origin & development of the Enteric Nervous System

The cells of the ENS originate from the neural crest in the developing embryo. Neural crest cells (NCC) are a migratory, multi-potent population of cells located at the apex of the neural tube, formed from the elevation and fusion of the two folds of the neural plate. NCC undergo an epithelial to mesenchymal transition and then delaminate to migrate from the neuroectoderm into the underlying mesenchyme. Following delamination, NCC migrate along a number of stereotypical pathways to form a wide range of tissues, including ganglia of the cranial nerves (V, VII, IX & X), the pia and arachnoid meninges, the adrenal medulla and the enteric nervous system (Sadler 1995).

Localisation of the origin of the specific NCCs responsible for the development of the ENS was initially determined using neural crest ablation studies (Yntema*, et al.* 1954, Peters-van der Sanden*, et al.* 1993, Burns*, et al.* 2001). In these studies selective ablations were performed at different levels of the neural crest of chick embryos and the effect on the formation of the ENS observed. These experiments were later supported by quail-chick chimaera studies performed by Le Douarin and colleagues. Le Douarin and Teillet engrafted sections of quail neural tube into the neural tube of a chick embryo at different somite levels and were able to track the migrating NCC, identifying those cells giving rise to the ENS. They demonstrated that the cells responsible for the development of the avian ENS were limited to the vagal neural crest adjacent to somites 1-7, along with small contributions from the thoracic and sacral region caudal to somite 28 in the lumbosacral neural crest (Le Douarin*, et al.* 1973, 1974, Burns*, et al.* 1998, Burns*, et al* 2001).

Study of NCC migration in the human fetus is limited by access to the developing gut. However studies using serial sections of aborted embryos and fetuses' have attempted to provide some answers. After delamination from the neural crest, cells migrate dorso-

ventrally along the course of the vagus nerve towards the primordial gut. They then congregate in the caudal branchial arches prior to moving into the anterior foregut at about 4 weeks gestation, colonising the distal hindgut by week 7.5 (Fu*, et al.* 2004, Wallace*, et al.* 2005). Initially, NCCs are located in the dorsal aspect of the gut and then migrate inwards and encircle the gut. As the colonisation continues, the ganglia group together forming the outer myenteric plexus starting in the oesophagus by week 7 and continuing in a craniocaudal direction to reach the distal hindgut by week 10. The submucosal plexus develops following a further, centripetal, migration of NCCs from the myenteric plexus, again following the same craniocaudal pattern, commencing in the foregut at week 8 gestation, but not reaching the distal hindgut until week 14 (Burns 2005, Wallace*, et al* 2005, Fu*, et al.* 2010). This apparent slowing of migration is due to a lengthening of the bowel rather than a decrease in velocity (Landman*, et al.* 2007).

The migration of NCCs through the developing bowel takes place in the form of a rapidly proliferating wave-front of NCCs at the head of a chain of cells left behind, which provides a scaffold to be populated by subsequent migrating and proliferating cells (Young*, et al.* 2004). There are two main factors involved in this process: the rapid cell proliferation at the migrating wave-front and chemotactic 'pull', e.g. GDNF and EDN3 produced by the developing mesoderm (considered in 1.1.4.). Landman *et al*, (2007) using a chick-quail chimaera gut explant coupled with mathematical modelling, suggested that rapid proliferation at the migrating wave-front is the predominant force responsible for colonisation, with NCCs preferentially colonising 'empty' aganglionic bowel ahead of the wave-front (Simpson*, et al.* 2006, Landman*, et al* 2007, Simpson*, et al.* 2007). They suggest that it is the discordance between the rate of proliferation relative to the rate of bowel growth that is responsible for the migration of NCCs along of the entire length of the bowel, and that any failure to colonise the entire length may either be due to rapid bowel

elongation, or inadequate proliferative properties of the NCCs in the wave-front. They have also shown a difference in the propensity to proliferate depending on the origin of the migrating NCCs in the neural crest (Zhang*, et al.* 2010) which suggests that both differences between NCCs and the local environmental factors within the gut determine ENS development.

Moreover, proliferation may not just be a driver for distal migration, but may also be responsible for the development of the myenteric and submucosal plexi. Zhang *et al*, (2010) have proposed a mechanism of hierarchical preference, suggesting that once the gut ahead is colonised, NCCs continue to proliferate and populate the existing plexus layer until the layer reaches its maximal cell density, after which proliferating cells will be driven inwards to populate the submucosal plexus (Zhang*, et al* 2010).

More recently, an elegant study by Nishiyama and colleagues provided evidence that the migration of mouse NCCs from the mid-gut to the hindgut also occurs via a transmesenteric route (Nishiyama*, et al.* 2012). Between 10-5-11.5 days post-coitum (DPC) the distal mid-gut and proximal hindgut lie in close proximity in a hairpin configuration. Using live cell imaging of individual cells it was shown that NCCs in the distal mid-gut migrate out and across the mesentery entering the hindgut at 12.5DPC. These cells were found to contribute to a significant proportion of NCCs populating the hindgut.

1.1.3. The sacral neural crest contribution

A relatively small population of neural crest-derived progenitor cells in the gut migrate from the sacral neural crest and have been shown to differentiate into neurons and glia in the distal hindgut. The evolutionary and functional impact of this population is uncertain: studies in chicks have found that only 17% of neurons in the myenteric plexus of the colorectum are derived from the sacral neural crest (Burns*, et al* 1998). Moreover, although

cells arising from the sacral neural crest may enter the hindgut in the absence of migration from the vagal neural crest, this contribution is not enough to preserve function (i.e. prevent a HSCR phenotype) (Burns*, et al.* 2000). It has been proposed that this may be either because the number of cells is simply not great enough, or that the development of a functional enteric plexus may be dependant on an interaction with the vagal NCCs (Burns*, et al* 1998, Burns*, et al* 2000).

1.1.4. Control of the developing ENS

In keeping with the complex process of NCC migration described above, a growing number of molecular signalling mechanisms have been shown to be involved in the regulation of the migration, proliferation, and differentiation of enteric NCCs.

1.1.4.1. Glial cell line-derived neurotrophic factor (GDNF)/ RET

The GDNF/RET signalling pathway is the most well studied pathway involved in the development of the ENS with a key role in development of neurons within the ENS. GDNF is expressed by the developing gut mesenchyme, whereas its transmembrane tyrosine kinase receptor RET (Abbreviation for 'rearranged during transcription') is found on the migrating NCCs themselves (Young, et al. 2001, Young, et al 2004). GDNF signalling has been shown to be necessary for enteric NCC differentiation, migration, survival and proliferation in the gut (Sanchez, et al. 1996, Peters, et al. 1998). In murine models a homozygous RET knockout results in complete intestinal aganglionosis distal to the oesophagus (Schuchardt, et al. 1995). In humans, mutations in RET have been implicated in 50% of familial cases of HSCR and up to 35% of non-familial cases (Attie, et al. 1995, Svensson, et al. 1998).

1.1.4.2. Endothelin-3 (ET3) / Endothelin type-B receptor (EDNRB)

Similarly to the GDNF/RET pathway, mesenchymal ET3 acts on migrating NCCs via the membrane receptor EDNRB, promoting both their proliferation and migration but inhibiting differentiation (Leibl*, et al.* 1999, Barlow*, et al.* 2003). Studies have shown that the time-dependent mesenchymal expression of ET3 is necessary for normal migration, and mutations in ET3 have been identified in both isolated and syndromic cases of HSCR, for example Shah-Waardenburg syndrome (Bidaud*, et al.* 1997). In mouse models ET3 and EDNRB knockouts produces a phenotype very similar to that usually seen in human HSCR as aganglionosis is largely restricted to the distal colon, in marked contrast to the widespread aganglionosis that results from a disruption of the GDNF/RET signalling pathway (Hosoda*, et al.* 1994).

As our understanding of the control and coordination of ENS development has deepened, there has been a corresponding growth in the number of downstream transcription factors and signalling pathways implicated (Table 1.1), such as the transcription factor Phox2b (Garcia-Barcelo*, et al.* 2003), the Notch signalling pathway (Okamura*, et al.* 2008, Ngan*, et al.* 2011), and Sonic Hedgehog (Ramalho-Santos*, et al.* 2000, Ngan*, et al* 2011). What is emerging is a complex relationship, with components effecting both synergistic and antagonistic roles at different developmental stages. It is this deepening understanding of how failures of normal development or signalling pathways may result in HSCR and the identification of "progenitor" cells that remain under the control of these transcription factors that has driven current research to develop new therapies aimed at improving ENS function within the bowel.

Table 1.2. Signalling pathways, transcription factors and genes involved in NCC development within the ENS

	Role in the developing ENS	References
GDNF/RET	• Promotes survival and proliferation • Promotes differentiate & migration	(Ivanchuk*, et al.* 1996, Sanchez*, et al* 1996, Peters*, et al* 1998)
ET3/EDNRB	• Promotes proliferation & migration • Inhibits differentiation	(Bidaud*, et al* 1997, Leibl*, et al* 1999, Barlow*, et al* 2003)
Sonic Hedgehog	• Promotes proliferation • Promotes concentric patterning	(Sukegawa*, et al.* 2000, Ngan*, et al* 2011)
Indian Hedgehog	• Promotes survival	(Ramalho-Santos*, et al* 2000)
Notch	• Promotes proliferation • Promotes glial development	(Okamura*, et al* 2008, Ngan*, et al* 2011)
Sox 10	• Progenitor maintenance	(Southard-Smith*, et al.* 1998, Kapur 1999, Lang*, et al.* 2000)
Sox 8	• Acts with Sox 10 • Progenitor maintenance	(Maka*, et al.* 2005)
Foxd3	• Regulates Sox10 • Progenitor maintenance	(Teng*, et al.* 2008)
Phox2b	• Promotes ENSPC survival • Regulates RET expression	(Young*, et al.* 1998, Pattyn*, et al.* 1999, Corpening*, et al.* 2008)
Hand2	• Promotes neuronal differentiation	(D'Autreaux*, et al.* 2007)
Netrin	• Required for centripetal migration	(Jiang*, et al.* 2003)
ASCL1 (Mash1)	• Promotes neuronal survival • Promotes mature differentiation	(Blaugrund*, et al.* 1996)
Pax3	• Regulates RET expression (with Sox10)	(Lang*, et al* 2000)
Zeb2	• Specification of vagal NCCs	(Garavelli*, et al.* 2003, Van de Putte*, et al.* 2003, Stanchina*, et al.* 2010)
TCF4	• Interacts with Wnt-ß-catenin pathway	(Zweier*, et al.* 2007)
Hoxb5	• Induces RET expression • Promotes migration	(Lui*, et al.* 2008, Carter*, et al.* 2012)

Table. 1.2. Abbreviations: DCC – detected in colorectal cancer *Table adapted from Obermayr, F et al. Nat Rev Gastroenterol.Hepatol 2013.* ***10****:43-57*

1.2. Intestinal Smooth muscle

The human gut is composed of two layers of smooth muscle, inner circular layer and outer longitudinal, however this configuration is altered in the colon where the outer longitudinal muscle is concentrated in three thin distinct bands (*taeniae coli*). Intestinal smooth muscle cells are smaller than the more common skeletal striated muscle. They contract at a slower rate to skeletal muscle, using the protein calmodulin rather than troponin (as in skeletal muscle) to regulate the influx of calcium to activate actin-myosin cross bridge cycling (Prosser 1974, Marston*, et al.* 1998). Furthermore, due to tight cell junctions and numerous cell membrane connections the whole intestinal muscle tube functions as a whole unit with spontaneous propagation of contractions distally (Kenton M. Sanders 2008).

1.2.1. Origin and development

As is seen in the migration of NCC in the developing bowel, myoblasts colonise the bowel in a rostrocaudal direction, reaching the oesophagus by embryonic week 8 and the hindgut by week 11. It takes a further three weeks for the distinct circular and longitudinal layers to develop through the gut (Fu*, et al* 2004, Wallace*, et al* 2005).

1.2.2. The development of gut motility

The process of propulsion of material through the intestine is complex, requiring interaction between three separate components: the enteric nervous system (ENS), the interstitial cells of Cajal (ICC) and the smooth muscle of the intestine. The study of its development within humans is limited. Three main types of propulsive waves are present within the gastrointestinal tract; segmentation waves, peristaltic waves and migrating motor complexes. Although, in the adult, the ENS is thought to be largely responsible for

these waves (Hasler 1999) both the ICC and intestinal smooth muscle are thought to exert their own influence on motility (K. M. Sanders 2006, Young 2008).

During fetal development each individual component, required for intestinal motility, is present by the 14th week of gestation. At this stage there are no functional propulsive waves but by the mid to late third trimester studies have observed fetal swallowing of amniotic fluid and the subsequent propulsion of the amniotic distally through the bowel (McLain 1963). Human studies on the later stages of development intestinal motility are largely limited to detailed observation utilising antenatal ultrasound. Initial studies observed fetal swallowing of amniotic fluid and the subsequent propulsion of fluid within the bowel in the late third trimester (McLain 1963). Gastric emptying has been shown to start at 24 weeks of gestation, although not exhibiting a mature pattern of contraction until nearer term (Sase*, et al.* 2005). Waves of contraction are not seen in the small intestine until 5 weeks later when small, infrequent coordinated waves may be observed (Goldstein*, et al.* 1987). There is little reported in the literature regarding colonic motility but studies looking at the human fetal internal anal sphincter have reported rhythmic contractions in the third trimester (Bourdelat*, et al.* 2001).

1.2.3. Intrinsic (myogenic) contraction

Intestinal smooth muscle retains the ability to continue to generate a rhythmic slow wave contraction in the absence of higher (CNS) control. These slow wave contractions are propagated cell-to-cell through calcium dependent voltage gated channels (at about -35mV). Although these waves are typically described as myogenic contractions, they are not initiated from smooth muscle cells, but rather has been shown that interstitial cells of Cajal (ICCs) provide the stimulus - acting as the 'pacemaker of the intestine'(Kenton M. Sanders 2008)(See 1.3.).

1.3. Interstitial Cells of Cajal

ICCs were first described in late 19th Century as cells situated between and distinct to smooth muscle cells and neurons within the gut (Ramon y Cajal 1911). However, their function was not determined until studies in the 1980's confirmed their responsibility for the 'pacemaker' activity of the gut (Bauer*, et al.* 1985, Hara*, et al.* 1986, Smith*, et al.* 1987).

1.3.1. Origin

ICCs have a common mesodermal origin with smooth muscle cells (Young*, et al.* 1996). The immature ICC expresses common smooth muscle markers, such as smooth muscle actin but can be distinguished histologically by the marked sparsity of contractile filaments and absence of desmin (K. M. Sanders*, et al.* 1999, Young 1999). Mature ICCs can be identified within the gut by their expression of the tyrosine kinase receptor (c-Kit) (Maeda*, et al.* 1992, S. M. Ward*, et al.* 1994). Human studies into the development of ICCs are limited, but studies suggest that they develop in parallel with myoblasts, colonising the bowel between week 8 and 11 gestation in a rostrocaudal direction. C-kit immunoreactivity is first detected in the outer muscle layers at week 9, and is almost fully developed by term (Kenny*, et al.* 1999, Wallace*, et al* 2005). In the mature bowel ICCs are predominantly located around the myenteric plexus but may also be seen throughout both the circular and longitudinal muscle layers (S. M. Ward*, et al.* 2000). The density of ICCs is also known to vary according to location within the bowel, with a marked reduction in c-kit expression seen in the colon compared to the small bowel (Mostafa*, et al.* 2010).

1.3.2. Function

In conjunction with both the ENS and intestinal smooth muscle ICCs are essential for coordinated peristalsis within the gut. Unlike their cardiac counterparts intestinal smooth muscle do not typically generate spontaneous electrical rhythmicity, rather it is now widely accepted that this is the main role of the ICC. Furthermore, they also play a role in signal transduction for both neural and mechanical stretch receptors inputs (S. M. Ward*, et al* 1994, Huizinga*, et al.* 1995).

The evidence supporting the pacemaker role of the ICC is strong: ICCs have been shown to possess spontaneous rhythmic electrical activity (S. M. Ward*, et al* 1994, Zhu*, et al.* 2009). The slow waves transmitted throughout both ICCs and intestinal smooth muscle are coordinated with the wave of contraction in ICCs slightly preceding that seen in the smooth muscle. Furthermore, studies have demonstrated electrical coupling between ICCs and smooth muscle cells (Dickens*, et al.* 1999, Kito*, et al.* 2003), although this has been harder to show on an ultrastructural level. It is important to note that the rhythmic slow wave contraction seen within the gut is essentially normal in the absence of NCC colonisation of the bowel (S. M. Ward*, et al.* 1999).

1.4. Notch signalling

Notch is a transmembrane protein responsible for regulating a number of developmental processes to control cell fate. It was first suggested to have a role in development the early 1900's where a homozygous mutation in the Notch gene was found to result in a 'notch' in the wing of Drosophila (Mohr 1919). However, it wasn't until the 1930's that its full significance in development was appreciated following experiments by Poulson which demonstrated that complete loss of Notch resulted in a lethal switch in the fate of cells from an epidermal phenotype to neural tissue (Poulson 1937). In vertebrates, there are currently four known Notch transmembrane receptors (Notch 1-4) and five corresponding transmembrane ligands (Delta-like 1, 3 and 4 and Jagged 1-2) which have been shown to have broad expression patterns throughout development (Lindsell*, et al.* 1996). Notch has been identified in most species with a high degree of conservation from *Caenorhabitis elegans* to humans (Ellisen*, et al.* 1991) and this is one factor that has driven research to identify its roles in development (e.g. cell differentiation, cell survival and apoptosis) which are increasingly being understood as highly context and cell-type dependent (Kadesch 2004).

1.1.5.1. The canonical Notch signalling pathway

The classical description of the mammalian canonical Notch pathway (Figure 1.2) is that activation occurs via contact with a Notch ligand (Delta/Jagged) expressed on adjacent cell membranes, which initiates a cascade of proteolytic cleavages of the Notch receptor resulting in the intracellular release of the notch intracellular domain (NICD) (Blaumueller*, et al.* 1997, Mumm*, et al.* 2000). This then crosses into the nucleus where it binds with recombination binding protein-J_K (RBP-J_K) (Jarriault*, et al.* 1995) changing its action from repressor to promoter of target genes transcription (e.g. the transcriptional repressor

families hairy enhancer of split [Hes] and hairy related transcription factor [HRT/Hey]) (Lai 2002, Kadesch 2004).

Although the canonical pathway has become increasingly well defined in recent years, so has the awareness of the complexity of the multiple interactions with pathways such as Sonic Hedgehog, tyrosine kinase receptors, Janus kinase- signal transducer and activator of transcription (JAK-STAT) and Wingless (Wnt) (Hurlbut*, et al.* 2007). Furthermore, it has also been demonstrated that Notch signalling can be activated in a NICD independent, non-canonical manner (Heitzler 2010). Thus, the complexity of Notch signalling is highly dependent on both context and the cell type involved.

1.1.4.2. Role of Notch in the central nervous system

There has been increasing interest in role of Notch since early studies reported that Notch is implicated in the regulation of both neuronal and glial differentiation, together with the maintenance of the neural progenitor pool (Gaiano*, et al.* 2000, Morrison*, et al.* 2000). Within the CNS it is thought that Notch is required for the maintenance of radial glia, which themselves function as neuronal and glial progenitors (Noctor*, et al.* 2001). During embryonic development, it has been shown that disruption of the key component of the canonical pathway RBPJ-κ results in a significant reduction in numbers of radial glia and corresponding increase in premature neurogenesis (Imayoshi*, et al.* 2010). Furthermore, if RBP-Jκ is knocked out in postnatal mouse models, it again leads to an increase of neurogenesis in the subventricular zone with corresponding loss of the neural progenitor pool (Gao*, et al.* 2009, Imayoshi*, et al* 2010). These results provide strong evidence for a crucial role for Notch signalling in both embryonic neural development and the post-natal maintenance of neural progenitors within the CNS.

Figure 1.2. The Canonical Notch Pathway

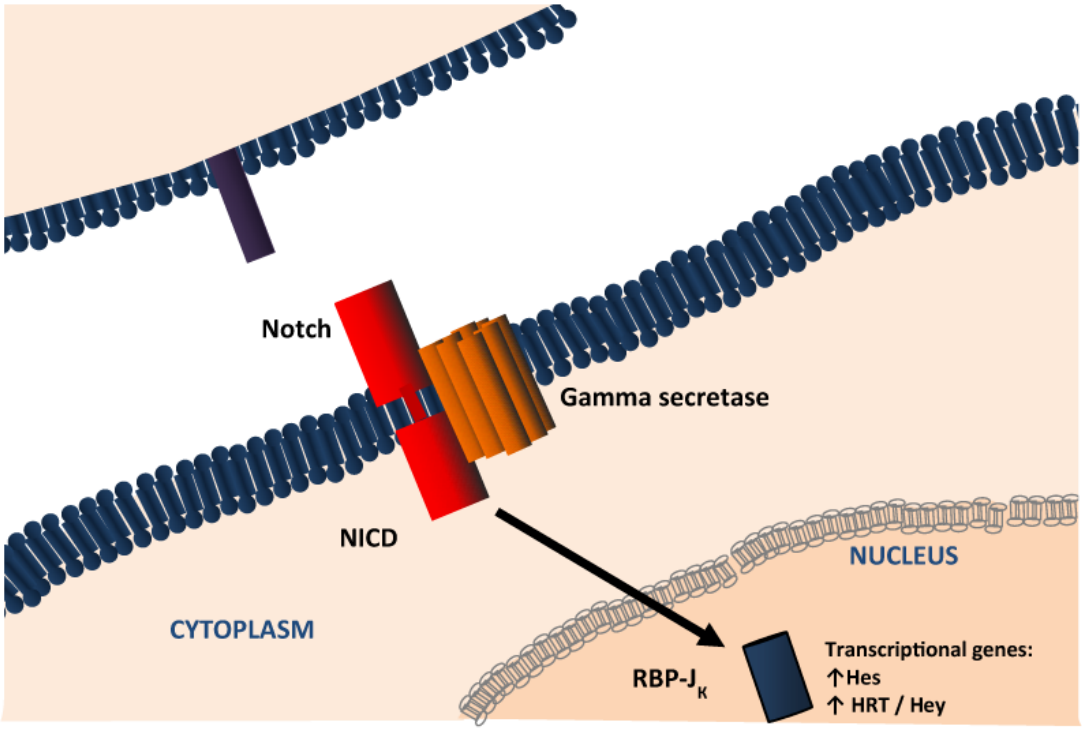


Figure 1.2. The Notch ligand delta is transported to the plasma membrane where it binds with the receptor and promotes the gamma-secretase mediated cleavage of the notch intracellular domain (NICD). This is then transported into the nucleus where it binds with RBP-J_K, thereby promoting the transcriptions of target genes, such as Hairy Expresser of Split (HES).

1.1.4.3. The role of Notch in the enteric nervous system

Compared to the comprehensive studies of Notch and its role in the CNS there have only been a limited number of studies specifically looking at its role in the ENS, and these are largely restricted to embryonic mouse models. Notch receptors and corresponding ligands have been shown to be present in the embryonic and post-natal enteric nervous system in both mice and rats (Sander*, et al.* 2004, Okamura*, et al* 2008). One study, the Pofut1 gene (involved in the posttranslational modification of the Notch receptor) was specifically deleted in NCCs using a Wnt1-Cre promoter (Okamura*, et al* 2008). Deletion of Pofut-1, thereby inactivating the Notch signalling pathway resulted in premature neurogenesis and a loss of ENS progenitors. More recently, this evidence has been strengthened using an alternative deletion of PTCH1 (a Sonic Hedgehog receptor), which was reported to generate

the same effects in a Notch dependent manner (Ngan*, et al* 2011). Given the similarities in the expression of markers between the CNS and ENS, and the preliminary data supporting its involvement in mouse ENS models it can be hypothesised that Notch is involved in the regulation of proliferation and neuronal differentiation in the post-natal human ENS.

1.5. Hirschsprung's disease and other Neuropathies of the Gut

1.5.1. Overview

Neuropathies encompass a wide range of conditions that are caused by a defect in cells arising from a neuronal lineage. Within the intestine these include diseases that affect the control systems of the gut, i.e. enteric neuropathies including HSCR, or the interfacing systems, i.e. anomalies in the ICC network which are implicated in conditions such as gastroparesis (Forster*, et al.* 2005). Enteric neuropathies may be either a primary defect in the neuronal cell line (summarised in table 1.3) or secondary to a systemic disease such as Crohn's disease, multiple endocrine neoplasia syndrome 2B or neurofibromatosis (Ferrante*, et al.* 2006, Hanemann*, et al.* 2007, Moline*, et al.* 2011). HSCR is the most common primary congenital enteric neuropathy and perhaps the most well studied.

Table 1.3. Summary of primary enteric neuropathies

Primary Enteric Neuropathies	Prevalence of histological evidence	Estimated lifetime prevalence	References
HSCR	Distal aganglionosis of variable length in 100%	1 in 5000	(Goldberg 1984)
Idiopathic achalasia	Progressive neuropathy, particularly nitrergic motor neurons	1 in 10 000	(Goldblum*, et al.* 1994, Goldblum*, et al.* 1996, Gockel*, et al.* 2008)
Idiopathic gastroparesis	Neuropathy identified in 70% with associated ICC depletion in 47%	1 in 4000	(Grover*, et al.* 2011)
Congenital chronic intestinal pseudo-obstruction (cCIPO)	Predominantly myopathies. 25% have associated neuropathy	1 in 10 000	(Schuffler*, et al.* 1977, Heneyke*, et al.* 1999)
Acquired chronic intestinal pseudo-obstruction (aCIPO)	Neuropathy identified in 60% of cases	<1 in 2000	(Knowles*, et al.* 2004, Lindberg*, et al.* 2009)

Table 1.3. Summary of primary enteric neuropathies, estimated prevalence of positive histological findings and lifetime prevalence. Adapted from Knowles et al. New perspectives in the diagnosis and management of enteric neuropathies. *Nat Rev Gastroenterol Hepatol 2013* ***10****(4):206-218*

1.5.3. Hirschsprung's disease

HSCR is the commonest congenital disorder of gut motility. Characterised by the absence of enteric ganglia, it typically affects the distal colon (rectosigmoid disease) in 80% of cases. However, the disease is variable in its manifestation and may involve longer segments such as total colonic HSCR (10% of cases) or even total intestinal HSCR in approximately 1% of children (Moore*, et al.* 2009, Ruttenstock*, et al.* 2009). The majority of infants born with HSCR will present within the first few days of life, usually with a delayed passage of meconium (>48hrs). Untreated, the tonic contraction of the affected bowel may quickly progress to a potentially life threatening bowel obstruction requiring urgent intervention.

The clinical entity was first described in the late 19th Century by the Danish physician Dr Harold Hirschsprung following the death of two young children patients (Hirschsprung 1887). However, although correctly surmising that the disease was due to a congenital defect in the development of the bowel resulting in obstruction he initial thought that the underlying pathology problem originated in the dilated segment of bowel. This unfortunately directed early surgical efforts towards resecting the normally innervated and dilated proximal bowel and leaving the effected distal colon behind.

It wasn't until the 1940's that the clinical presentation of HSCR was attributed to an absence of ganglia in the distal colon (Tiffin*, et al.* 1940, Whitehouse*, et al.* 1948). Shortly after this Orvar Swenson and Alexander Bill reported their initial results following resection of the contracted distal bowel and 'pull-through' of the proximal bowel (Swenson*, et al.* 1948), which as a concept still underpins the modern treatment of HSCR today. From the 1950's onwards mortality associated with HSCR has dropped dramatically, with current overall survival rates in England reported at over 90% (Best*, et al.* 2012).

1.5.3.1. Epidemiology and aetiology of HSCR

HSCR has an overall worldwide reported incidence of 1 in 5000 live births (Goldberg 1984), however most large scale epidemiological studies have been limited to Caucasian populations. There is evidence to suggest that there is significant variation within interracial groupings from 15:100 000 live births in European populations up to 28 in 100 000 in Asian groups, which is consistent with the prevalence of known genetic mutations in the respective populations (Emison*, et al.* 2005, Emison*, et al.* 2010). In addition to the racial variation HSCR is also associated with a significant male:female preponderance (4:1)(Emison*, et al* 2005). Associated congenital anomalies can be found in between 4 and 35% of cases (summarized in table 1.4).

Table 1.4 Congenital anomalies found in association with HSCR

Associated Neural Crest Anomalies			
Sensorineural	• Congenital central hypoventilation syndrome		
Deafness	• Isolated sensoriuneural deafness		
	• Type IV (Shah) Waardenburg Syndrome		
Cardiovascular	• DiGeorge Syndrome		
Cleft Palate	• Goldberg Sphrintzen Syndrome		
	• DiGeorge Syndrome		
Systemic anomalies	• Multiple endocrine neoplasia type IIA and IIB		
	• Neurofibromatosis Type I		
	• Smith-Lemli Opitz syndrome		
Other Anomalies			
• Trisomy 21	• Small Bowel Atresia	• Duodenal Atresia	
• Microcephaly	• Mental retardation	• Undescended testes	
• Inguinal hernias	• Imperforate anus	• Colonic atresia	

Table 1.4. Additional congenital anomalies found in association with Hirschsprung's disease. Adapted from (Kenny*, et al.* 2010)

Although all cases of HSCR arise from a failure of the ENS to migrate and populate the entire bowel during development the precise underlying aetiology of HSCR remains to be fully elucidated. It is widely accepted that HSCR has a multi-genic disorder with the majority of cases arising from sporadic mutations (Emison*, et al* 2005). However, more recent evidence suggests that environmental factors may also play a contributory role in the development of this disease (N. L. Ward*, et al.* 2012, Lake*, et al.* 2013).

1.5.3.2. HSCR Genetics

The majority (70-80%) of cases are thought to be sporadic, but familial forms do exist, albeit with variable penetrance accounting for approximately 20% of cases (Amiel*, et al.* 2008). Mutations in genes involved in any one of the pathways previously described (see 1.1.4) may result in HSCR. However, individuals may possess significant mutations in these key genes and yet not display any phenotypic features of the disease, which may indicate a significant role for environmental factors in the development of HSCR (Kenny*, et al* 2010).

The most studied gene in relation to susceptibility to HSCR is RET, mutations in RET can be found in more than 50% of familial cases of HSCR and 15-20% of sporadic (Amiel*, et al* 2008). Furthermore, linkage studies looking at previously un-investigated non-coding regions of the RET gene suggest that a mutation in RET (coding or non-coding) may be necessary for the development of the HSCR phenotype in almost all cases. However these studies also confirmed that RET mutations are not sufficient alone, in all cases, to result in the disease (Emison*, et al* 2010).

In a family with one affected child the risk of a subsequent sibling being affected lies between 1 and 33% depending on the length of aganglionic bowel and the sex of the children. The highest recurrence risk for siblings is 33% for male children with a sister with long segment disease, falling to just 1 % for females with an older affected brother (Amiel*, et al* 2008). The underlying reason for this variable, sex dependent penetrance is not yet fully understood. Genetic screening studies in mice have identified a number of genes expressed in the developing ENS that are linked to the X-gene but no human studies have identified a locus on the X-gene (Heanue*, et al.* 2007). Alternative hypotheses suggest that differential epigenetic regulation at the RET locus may account for these changes (Amiel*, et al* 2008).

1.5.3.3. Current surgical management

The distal bowel obstruction, due to the tonic contraction of aganglionic bowel can usually be resolved with rectal washouts and dilatations in newly diagnosed infants. This allows a definitive single-stage procedure to be performed electively in the first few months of life (Keckler*, et al.* 2009). If adequate decompression of the bowel is not achievable with these simple measures a levelling stoma is required in the early neonatal period.

There are a number of surgical techniques currently in widespread use, however they all share the key aim of resecting the majority of the aganglionic bowel and pulling down 'normal' ganglionic to a low anastomosis above the anal sphincters. Approaches included both single and multi-staged procedures and both laparoscopic and open approaches. The two most popular techniques in widespread use are the Duhamel and modified Soave pull-throughs, however, there is a paucity of evidence to promote a single technique as the operation of choice (Baillie*, et al.* 1999, Conway*, et al.* 2007, Craigie*, et al.* 2007).

1.5.3.4. Long-term Outcomes

Over recent decades, the surgical management of HSCR has continued to evolve; however, despite adoption of minimally invasive approaches and refinements in surgical technique, HSCR is still associated with significant long-term morbidities. A number of cohort studies report significant rates of refractory constipation and fecal incontinence affecting between 10% and 30% of patients, resulting in significant social morbidity (Ludman*, et al.* 1995, Baillie*, et al* 1999, Minford*, et al.* 2004, Mills*, et al.* 2008). Furthermore, one recent long-term Finnish study has also suggested that rates of certain morbidities such as fecal incontinence may actually increase with time (Jarvi*, et al.* 2010). It is the results of studies such as these, coupled with our growing understanding of the development of the enteric nervous system (ENS), that have provided the stimulus to investigate the possibility of

developing alternative or adjunctive therapies to surgery in an effort to alleviate some of the ongoing morbidity experienced by children and adults with HSCR.

1.6. Development of potential cell based therapies for HSCR

Advances, particularly over the last 15 years, in our understanding of the development of the ENS and in translational stem cell research has prompted growing interest into the potential of using cell-based therapies to 'replace' the missing components of the ENS with the goal of improving the quality of life of patients with enteric neuropathies such as HSCR.

1.6.1. Sources of stem/progenitor cells

Following the discovery that neurogenesis is a continuous process in the adult human brain and not just restricted to early development (Eriksson*, et al.* 1998) there has been interest in the potential of using neural progenitor cells to restore defects in ENS colonisation. This has driven the efforts of several research groups towards the development of both *de novo* and adjunctive cell-based therapies for HSCR. The underlying hypothesis for these therapies is that progenitor cells exist in the post-natal gut, and that they retain the potential to differentiate into both enteric glia and neurons. Furthermore, these progenitors can be isolated and expanded in-vitro to generate sufficient numbers for transplantation with the goal of restoring a normal pattern of bowel contractility and/or improving function. Consequently, a number of potential sources have been investigated, including the central nervous system (CNS), embryonic stem cells (ESC) and the ENS itself.

1.6.1.2. The central nervous system

The potential for using progenitors derived from the CNS was demonstrated in early studies, transplanting CNS derived progenitor cells into the pylorus of a well-established mouse model of gastroparesis (a neuronal nitric oxide synthase (nNOS) neuron null mouse). Within one week of transplantation new functional nNOS neurons were seen within the muscle wall and this was associated with a significant improvement in both relaxation of

the pylorus and gastric emptying (Micci*, et al.* 2005). This study provided the first strong evidence to support the use of CNS-derived transplanted progenitors in the treatment of gastrointestinal motility disorders. More recent studies have looked specifically at functional outcomes in an in-vivo rat model of HSCR. In this study Dong and colleagues report that new nNOS and choline acetyltransferase (ChAT) positive neurons could be seen following transplantation of rat fetal CNS-derived progenitors into the aganglionic rectum of a rat, and more importantly that this transplantation could restore the rectoanal inhibitory reflex (Dong*, et al.* 2008). However, there are both practical and ethical issues regarding the isolation and transplantation of fetal human CNS progenitors that would pose significant barriers to development of a clinical application.

1.6.1.2. Human embryonic stem cells

Studies have also demonstrated the ability to direct the differentiation of human ESC (HESC) and progenitor cells isolated from amniotic fluid towards a neuronal lineage, including the neuronal phenotypes found within the ENS (Hotta*, et al.* 2009, Kamiya*, et al.* 2011, Bottai*, et al.* 2012). Progenitors derived from HESC and induced to express neural crest markers have been transplanted into embryonic mouse gut explants and shown to have the capability to migrate and differentiate into cells expressing markers of both neuronal and glial phenotypes (Hotta*, et al* 2009). However, the translation from promising animal study to human trial has been hampered by similar issues to those limiting the clinical application of CNS-derived stem cells.

1.6.1.3. The enteric nervous system

Despite the clear ability of progenitors from a variety of lineages to migrate, differentiate and even show functional improvement post-transplantation the mainstay of current research is focused around the use of ENS-derived progenitor cells (ENSPC). This source of progenitors has the key advantage of providing the ability to perform autologous transplantations, thus alleviating the necessity of immunosuppression and avoiding some of the technical and ethical concerns associated with some of the alternative sources.

1.6.2. Potential role of antenatal intervention

The prospect of antenatal intervention with the aim of correcting the failure to colonise the distal bowel in HSCR is attractive. Studies have reported that in an experimental model of aganglionosis, created by ablating of the vagal NCCs before they have migrated, a 'rescue' may be achieved following transplantation of a small number of NCCs into the existing migratory pathways (Druckenbrod*, et al* 2005). The potential of antenatal intervention, either with transplantation or by manipulation of the known migratory signalling pathways is certainly attractive however research into the development of antenatal therapies is not a major active field of research primarily due to the inherent difficulties of diagnosing HSCR in the antenatal period. Although there are known genetic mutations, in particularly in the Ret-GDNF and ET-3-EDNRB genes, the genetic picture is far from simple with an ever-growing number of associated single or multiple mutations being identified. Furthermore there is wide variability in the penetrance of these known mutations, such that even in individuals with mutations known to be strongly associated with HSCR we are unable to accurately predict whether they will develop a HSCR phenotype. This might suggest that other factors, such as environmental insults during gestation are also involved in the development of HSCR. However, it also makes a widespread genetic antenatal screening programme non-viable at this current time.

The alternative antenatal diagnostic modality is the use of ultrasonography however this has yet to be shown to be of much value. Not least because colonic dilatation is rarely observed before 25 weeks (Belin*, et al.* 1995) making it highly improbable that features of bowel obstruction will be visible at the time of routine antenatal scans. Furthermore, abdominal distension due to bowel dilatation is a postnatal phenomenon, appearing after the first few days of life. Therefore, largely due to the lack of a reliable method of diagnosing these children in the antenatal period the prospects for fetal intervention remain minimal.

1.6.3. Enteric nervous system progenitor cells

1.6.3.1. Isolation of ENSPC

The ability to isolate ENSPC was initially reported using fetal bowel from mice and rats. It was found that a subpopulation of cells isolated from dissociated gut tissue expressing the neural crest markers such as P75 could be maintained in culture, forming conglomerations of cells termed neurospheres. The subsequent demonstration that cells could be isolated from mature post-natal gut, and that these cells maintain the potential to differentiate into enteric neurons and glia prompted further study into a potential therapeutic role for these cells (Natarajan*, et al.* 1999, Sidebotham*, et al.* 2002).(Kruger*, et al.* 2002)

Importantly, with regard to obtaining a practical source for future therapies it has since been shown that ENSPC can be isolated from human postnatal bowel including patients with HSCR (Rauch*, et al.* 2006, Almond*, et al.* 2007, Lindley*, et al.* 2008, Metzger*, et al.* 2009). Cells can be isolated either from full thickness colonic wall biopsies (Almond*, et al* 2007, Lindley*, et al* 2008) or as described by Metzger *et al*, from endoscopic gut mucosal biopsies (Metzger*, et al* 2009). Significantly, human cells, including those isolated from patients with HSCR have been transplanted into both embryonic aganglionic mouse and human postnatal colon and have been shown to migrate, differentiate (Lindley*, et al* 2008, Metzger*, et al* 2009). Furthermore, Lindley *et al,* demonstrated that human cells from patients with HSCR maintain the ability to restore a normal pattern of contractility to embryonic aganglionic bowel (Lindley*, et al* 2008).

1.6.3.2. In-vitro culture of ENSPC

Neural progenitors both from the CNS and the ENS are typically maintained in culture in the form of neurospheres. However, despite the widespread use of neurosphere assays in CNS neural progenitor research our knowledge of the precise composition and behaviour of ENS neurospheres is limited. Studies have shown that ENS-derived neurospheres contain a mixed population, comprising of both progenitors and more differentiated cell types such as neurons and glia (Kruger*, et al* 2002, Almond*, et al* 2007). Later studies have demonstrated that ENSPC cultured as neurospheres are capable of clonal expansion itself a key property for a stem cell population (Lindley*, et al.* 2009), but more importantly, when transplanted onto embryonic aganglionic gut explants ENSPCs proliferate and differentiate into cells expressing neural and glial markers (Natarajan*, et al* 1999, Bixby*, et al.* 2002, Sidebotham*, et al.* 2002, Bondurand*, et al.* 2003, Lindley*, et al* 2008) However, how cells behave whilst in this artificial environment particularly in terms of proliferation and differentiation, and the control of these processes remains largely unknown.

1.6.3.3. Origin of ENSPC

Despite the significant progress in the isolation and characterisation of ENSPC in-vitro the identity and localisation of neural progenitors in-vivo is yet to be determined. The common assumption is that ENSPC present in the post-natal bowel are derived from the vagal NCC colonising the bowel during development. Current evidence, supported by the expression of glial markers (GFAP, Sox10, S100) and lineage tracing studies, is stacking up behind a glial lineage for these cells (Joseph*, et al.* 2011, Laranjeira*, et al.* 2011), however the absence of a definitive marker for ENSPC has hampered their identification in-vivo.

With regard to localisation, early studies have suggest that progenitors are located in the region of the myenteric plexus, within the enteric ganglia themselves (Kruger*, et al* 2002), which explains their absence in the distal aganglionic bowel in HSCR. Not all studies agree with this, a more recent pulse-chase DNA labelling study from the Gershon group suggested that a small pool of progenitors exist in an extra-ganglionic location giving rise to cells that subsequently migrate into the myenteric and submucosal ganglia of the postnatal gut in-vivo (Liu*, et al.* 2009). However, the current inability to reliably identify ENSPC in-vivo has prevented any definitive conclusions being drawn.

1.6.4. Postnatal ENSPC-based therapies

Studies confirming the ability of human postnatal ENSPC to restore a normal pattern of contraction to an aganglionic model have provided significant stimulus in the search to develop cell-based therapies as a treatment modality for HSCR (Lindley*, et al* 2008). However transplantation into a small section of embryonic bowel in-vitro is an entirely different proposition to transplanting these ENSPC into mature postnatal human gut in-vivo. The ENS is a complex and extensive division of the nervous system and even in HSCR limited to the sigmoid colon both the population of transplanted ENSPC that would be required to restore the ENS and the distances required for them to migrate would be vast. This has prompted some groups to target the development of adjunctive therapies to surgery rather than aiming to transplant ENSPC into large lengths of aganglionic colon. The underlying premise being that surgery always leaves behind a small cuff of aganglionic tissue around the internal anal sphincter and that conceivable that it this residual cuff that is responsible for at least some of the long-term morbidity seen. It is hoped that the more practically achievable target of transplantation of ENSPC into this defined region may improve current outcomes (Almond*, et al* 2007, Lindley*, et al* 2008).

Although the majority of studies looking at the potential role for ENSPC based therapies have been based in ex-vivo models, a recent study has reported the ability to transplant post-natal ENPSC into mice. They have shown that ENSPC can migrate extensively into postnatal mouse bowel, survive, proliferate, and integrate with the host's nervous system. Most importantly, these transplanted cells can differentiate into electrophysiologically active neurons (Hotta*, et al.* 2013). Although encouraging, it still remains to be seen whether transplanted cells can actually achieve 'restoration of function' and questions remain regarding the ability to translate the findings of these studies to humans.

1.6.5. Outstanding questions

1.6.5.1. Is transplantation of autologous ENSPC safe?

Since the main aim of any new HSCR therapy is to alleviate morbidity rather than mortality it is unsurprising that ensuring the safety of any future cell-based therapy is of primary importance. Given the dramatic change in behaviour seen when these cells are isolated from the human colon where they move from what appears to be an essentially quiescent state to that of rapid proliferation in-vitro it is important to understand the mechanisms that regulate this change. The key question being, what will happen to this behaviour when they are transplanted back into the environment of a human colon, will they come back under the control of the local regulatory mechanisms or will they continue to proliferate and develop malignant potential? Detailed long-term studies are required to determine the how ENSPC behaviour alters post-transplantation, specifically there is a need to assess the genomic stability of these cells to establish what tumour potential they may pose.

1.6.5.2. Will transplanted ENSPC survive in human post-natal aganglionic bowel?

Since NCC colonisation of the gut involves a complex relationship between both the migrating NCC and the developing gut, it is possible part of the underlying cause of the failure to colonise the distal bowel may continue to make aganglionic inhospitable to ENSPC transplantation. It has been reported that the ability of ENPSC becomes more limited simply by the maturation of the bowel (Druckenbrod*, et al.* 2009) so it is quite possible that a post-natal aganglionic environment may make it impossible for cells to migrate further, differentiate or even survive. However, recent work presented by two groups: Hotta *et al*, and Cooper *et al*, provides at least some hope that these obstacles may be overcome, in preliminary studies transplanting ENPSC into postnatal mice both groups have shown that the transplanted cells continue to migrate differentiate and even integrate with the host

ENS (Cooper*, et al.* 2012, Hotta*, et al* 2013). Even though these early results continue to provide support for the feasibility of future cell-based therapies it still one step further to transplant ENSPC into an aganglionic environment. As other groups have suggested it may require manipulation with the addition of missing neurotrophic factors such as GDNF or ET3 to either the recipient's bowel or the pre-transplanted ENSPC themselves for integration with the bowel to be successful (Bondurand*, et al.* 2006, Schafer*, et al.* 2009).

The possibility of manipulating aganglionic bowel to optimise the environment for receiving transplanted ENSPC also raises the question as to whether manipulation of the environment may allow inward migration of neurons and glia arising from progenitor cells in neighbouring ganglionic regions in-vivo (Laranjeira*, et al* 2011). The potential for neurogenesis following injury to the bowel has been demonstrated in studies in both mice and guinea pigs (Matsuyoshi*, et al.* 2010, Laranjeira*, et al* 2011). However, it has not been universally possible to replicate their success with the Morrison group unable to demonstrate any neurogenesis in-vivo (Joseph*, et al* 2011). Although this area of research merits further study any therapy that involves the manipulation of proliferation of cells in-vivo has additional potential for 'off-target' effects, including the proliferation of unexpected cell types and their consequences.

1.6.5.3. Which cell type will provide the best functional response?

The neurosphere is a conglomeration of progenitors, glia, neurons and smooth muscle and as yet we are still unable to find a specific marker to identify the progenitor cells within this melee. Recent studies have suggested that neural progenitors may arise from cells with a glial lineage. This is based on in-vitro work demonstrating that cells expressing the glial marker GFAP have the capability to differentiate into functioning neurons (Joseph*, et al* 2011, Laranjeira*, et al* 2011). This is also consistent with studies looking at the developing CNS, which have shown that cells expressing the astrocyte/glial marker GFAP give rise to neurons (Garcia*, et al.* 2004). However, these findings have yet to be consistently repeated in-vivo within the ENS (Joseph*, et al* 2011). Furthermore what we have yet to ascertain is whether it is the mature glia within the bowel that under the right conditions revert back to a more 'stem' cell-like behaviour, or whether the progenitor cells are a separate population themselves which also express glial cell markers. Even when the identity of the ENSPC is resolved we will still need to ascertain whether it is more effective to transplant a purified sub-population of ENSPC or fully differentiated neurons, or indeed whether it needs to be the mixed population contained within neurospheres. Studies are needed to determine which of these cell types will provide the best functional outcome and more importantly which type will be the safest?

1.6.5.4. What is the most effective route to deliver cells?

Aside from optimising the behaviour of ENSPC and the recipient's colon there are also more practical issues that need to be considered before bringing these therapies into clinical trials. Such as, what is the most effect method of delivery for transplanted ENSPC? Is it most effective to transplant ENSPC in their cultured form as neurospheres, or should they be dissociated first and injected as a cell suspension? Where is the optimal site for delivery, should we be aiming to transplant them in to the region of the sub-mucosal plexus, the myenteric plexus or elsewhere. Preliminary data is available from early animal transplantation studies, which either place transplanted cells directly into muscular wall of the gut or simply inject cells directly into the peritoneum. Of these two techniques direct placement into the gut wall itself appears to be more promising because although ENSPC injected into the peritoneum were found to have started to differentiate into neurons and glia none were identified in the aganglionic colon of the mouse (Micci*, et al* 2005, Tsai*, et al.* 2010). In addition to these techniques there remains the possibility of using microinjection, delivering cells either endoscopically or under ultrasound-guidance. These microinjection techniques may be of particular benefit for therapies targeting residual aganglionic bowel adjacent to the internal anal sphincters. What is needed is a systematic study using the same animal model to compare delivery methods in terms of the specificity of their delivery to the colon and the subsequent integration with the host environment and function response.

1.7 Aims of the thesis

The aims of this thesis were largely to address the first two questions outlined in 1.6.5. Firstly, is transplantation of autologous ENSPC safe and secondly, will transplanted ENSPC survive in human post-natal aganglionic bowel? In order for ENSPC-based transplantation therapies to progress towards clinical trials we need a better understanding of what these cells are and how they behave. As well as the control mechanisms responsible for the change in behaviour seen during the transition from in-vivo to in-vitro and again following transplantation. Specifically the original aims of this thesis were to:

1. Determine the behaviour, with specific regard to proliferation of both mouse and human-derived ENSPC in-vitro.

2. Determine the role of the Notch signalling pathway in the regulation of this behaviour.

3. Develop an ex-vivo model of postnatal human aganglionic colonic smooth muscle in which the behaviour and functional effect of transplanted ENSPC can be investigated.

While carrying out experiments to meet the third aim an unexpected observation was made, which brought about the fourth and final aim:

4. To determine whether neural progenitors are present within the aganglionic bowel of children with HSCR and whether these cells have the same functional potential as those isolated from normal ganglionic bowel.

Chapter 2: Materials and Methods

2.1. Standard sample isolation techniques

2.1.1. Embryonic mouse

A Home Office project license was not required for the work detailed within this thesis as no regulated procedures were performed. Female CD1 mice (Charles River Laboratories, Kent, UK) were time mated and sacrificed under UK Home Office Schedule 1 conditions by CO_2 asphyxiation at 11.5 days post coitum (dpc). Vaginal plug = 0.5dpc. A laparotomy was performed under sterile conditions, using 70% (v/v) ethanol as a disinfectant, at which time the peritoneal cavity was opened, uterus excised en-block and placed in warmed high glucose (4.5g/l) Dulbecco's modified Eagle medium with L-glutamine and sodium pyruvate (DMEM, Life Technologies, Paisley, UK). 9cm plastic petri dishes prepared with 25ml Sylgard® 184 (Dow Corning, Midland MI, USA) were used as dissection plates and sterilised using 70% (v/v) ethanol and exposure to UV light for 30 minutes. Each embryo was removed from the uterus intact and the decapitated before proceeding further. Dissection was performed aseptically using a dissection microscope (M165FC, Leica Microsystems, Bucks, UK) to remove any attached mesentery and to isolate the desired region of bowel (Fig 2.1). Resected specimens were kept for a maximum of 2h in high glucose DMEM at 37°C supplemented with 10ng/ml gentamycin, 100 Units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies) until required.

2.1.2. Adult and neonatal mouse

Adult CD1 mice or postnatal day 1 pups from female CD1 mice (Charles River Laboratories) were sacrificed under Schedule 1 by CO_2 asphyxiation and cervical dislocation. Dissection was performed under the same conditions as embryonic mice. A laparotomy was performed, the colon removed intact and kept as per embryonic bowel until required.

2.1.3. Postnatal human

Ethical approval was obtained from the North West 3 Research Ethics Committee (Ref:10/H1002/77) to collect human colonic samples during elective surgical procedures. Individual consent was obtained from the parents of each child recruited into the study and included samples from both patients with Hirschsprung's disease (HSCR) and controls, i.e. those undergoing either the formation or closure of colostomy for pathology not thought to affect the innervation of the bowel (Table 2.1). Samples were anonymised before processing and all human tissue was both stored and destroyed according to guidelines set down in the Human Tissue Act 2004. $1cm^2$ specimens of bowel were taken from both the ganglionic and aganglionic regions of the resected colon – confirmed by an absence of ganglion cells on fresh frozen sections examined by a consultant paediatric pathologist intraoperatively. A small 2mm section was immediately fixed in 4% (w/v) paraformaldehyde, and the remaining specimen was transported back to the laboratory, wrapped in sterile saline soaked gauze on ice at 4°C within 4 hours.

Figure 2.1 Dissection of Embryonic mouse bowel

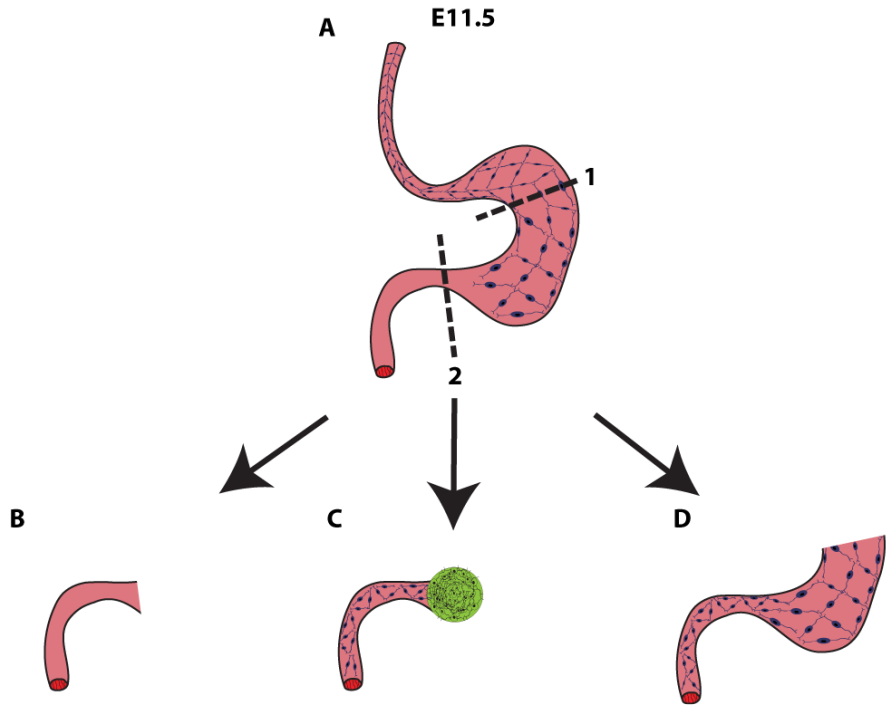


Figure 2.1. (**A**) Embryonic murine colon +/-caecum were harvested at E11.5 at which time the wave front of migratory ENS progenitors will have just reached the caecum(Druckenbrod*, et al.* 2005). For generation of embryonic neurospheres the caecum was removed intact. Ganglionic specimens (**D**) for transplantation experiments were created by dividing the caecum at **A1**. Aganglionic samples were taken at **A2**, distal to the migratory wave front and either cultured alone (**B**) or transplanted with a neurosphere at the proximal end (**C**). Any mesentery attached to the bowel was removed.

Table 2.1. Underlying pathology and age of study recruits.

Patient ID	Pathology	Age at Operation	Gestation	Successful progenitor cell isolation	
				Ganglionic Bowel	Aganglionic Bowel
H001	Hirschsprung's – Short segment	1 month	Term	Yes	Not attempted
H002	Hirschsprung's – Short segment	1 month	Term	Yes	Not attempted
H003	Hirschsprung's – Short segment	2 months	Term	Yes	Not attempted
H004	Hirschsprung's – Short segment	2 months	Term	Yes	Not attempted
H005	Hirschsprung's – Short segment	1 month	Term	Yes	Not attempted
H006	Hirschsprung's – Short segment	4 months	Term	Yes	Not attempted
H007	Hirschsprung's – Short segment	1 year	36	Yes	Not attempted
H008	Hirschsprung's – Short segment	2 months	Term	Yes	Not attempted
H009	Hirschsprung's – Short segment	1 month	Term	Yes	Not attempted
H010	Hirschsprung's – Short segment	2 months	Term	Yes	Not attempted
H011	Hirschsprung's – Short segment	2 months	Term	Yes	Not attempted
H012	Hirschsprung's – Short segment	3 months	Term	Yes	Not attempted
H013	Hirschsprung's – Short segment	6 months	Term	Yes	Yes
H014	Hirschsprung's – Short segment	3 months	Term	Yes	Yes
H015	Hirschsprung's – Short segment	3 months	Term	Yes	Yes
H016	Hirschsprung's – Long segment	3 months	Term	Yes	Yes
H017	Hirschsprung's – Short segment	3 months	Term	Yes	Yes
H018	Hirschsprung's – Short segment	3 months	Term	Yes	Yes
H019	Hirschsprung's – Short segment	1 year	Term	Yes	Yes
H020	Hirschsprung's–Ileal/Total colonic	2 weeks	Term	Yes	No
H021	Hirschsprung's – Short segment	5 weeks	Term	Yes	Yes
H022	Hirschsprung's – Short segment	4 months	Term	Yes	Yes
H023	Hirschsprung's – Total colonic	3 weeks	Term	Yes	No
C001	Anorectal Malformation	1 year	Term	Yes	N/A
C002	Gastroschisis	2 months	35	Yes	N/A
C003	Anorectal Malformation	1 day	Term	Yes	N/A
C004	Constipation – Sigmoid colectomy	5 years	Term	Failed	N/A
C005	Constipation – Sigmoid colectomy	16 years	Term	Failed	N/A
C006	Anorectal Malformation	6 months	Term	Yes	N/A
C007	NEC – Closure of stoma	3 months	29	Yes	N/A
C008	Anorectal Malformation	3 months	Term	Yes	N/A
C009	Isolated colonic perforation	6 months	28	Yes	N/A

Table 2.1. Successful progenitor cell isolation is taken as the formation of neurospheres within 28 days of culture. Term gestation is taken as birth a between 37 and 42 weeks gestation. The gestational age of all births outside this range are given.

2.2. Standard buffers and tissue culture media

2.2.1. Standard neurosphere medium (SN Medium)

DMEM low (1% w/v) glucose, (Gibco®, Life Technologies, Paisley, UK), supplemented with:

- 2% v/v chicken embryo extract (Sera Laboratories, West Sussex, UK)
- 1% v/v Fetal calf serum (Sigma-Aldrich, Dorset, UK)
- 2mM final concentration L-glutamine (Gibco®)
- 100 Units/ml Penicillin / 100 µg/ml streptomycin
- 0.05mM final concentration mercaptoethanol (Gibco®)
- 20ng/ml final concentration fibroblast growth factor II (Source Bioscience, Nottinghamshire, UK)
- 20ng/ml final concentration Epidermal growth factor (Sigma-Aldrich)

2.2.2. Fetal Bovine Medium (FB Medium)

DMEM high glucose (4.5% w/v, Gibco®), supplemented with:

- 10% v/v Fetal calf serum
- 2mM final concentration L-glutamine
- 100 Units/ml Penicillin / 100 µg/ml streptomycin

2.2.3. Horse Serum Medium (HS Medium)

DMEM high glucose (4.5% w/v), supplemented with:

- 20% v/v Medium 199 (Gibco®)
- 7% v/v Heat inactivated horse serum (Gibco®)
- 100 Units/ml Penicillin / 100 µg/ml streptomycin

2.2.4. Tissue Culture Medium (TC Medium)

DMEM high glucose (4.5% w/v), supplemented with:

- 1% v/v Fetal calf serum
- 2mM final concentration L-glutamine
- 100 Units/ml Penicillin / 100 µg/ml streptomycin
- 0.05mM final concentration mercaptoethanol

2.2.5. Immunofluorescence Blocking Buffer (IBB)

PBS ($CaCl_2$ / $MgCl_2$ free, Gibco®) supplemented with:

- 20% v/v Goat serum (Gibco®)
- 0.1% w/v NaN_3 (Sigma-Aldrich)
- 0.1% v/v Triton X (VWR, Leicestershire, UK)
- 1% w/v Bovine serum albumin (Sigma-Aldrich)

(For staining of cell surface receptors 0.1% triton was replaced with 0.1% Saponin (Sigma-Aldrich) to preserve the architecture of the cell membrane.)

2.3. Cell Culture Techniques

2.3.1. Generation of neurospheres from primary cultures

The techniques for generating neurospheres from both mouse and human sources were based on previously published work from our group (Almond*, et al* 2007, Lindley*, et al* 2008).

2.3.1.1. Embryonic mouse

To generate a single cell suspension E11.5 mouse caeca (2.1.1) were incubated with 0.05% (w/v) trypsin (Sigma-Aldrich) at 37°C for 1h. The sample was then centrifuged at 150g for 5 minutes, supernatant removed and 1ml of SN culture medium added before mechanically dissociating the suspension by trituration using a 1000µl pipette. The resultant single cell suspension was counted using a haemocytometer and aliquots of 2-5x10^5 cells transferred to non-adherent 35mm dishes (530588, Corning BV Life Sciences, Amsterdam, Netherlands). Additional standard neurosphere (SN) medium was added to reach a final volume of 6ml/dish and dishes kept in a Sanyo MCO humidified incubator at 37°C and in the presence of 5% CO_2 (Panasonic Biomedical, Loughborough, UK). SN medium was supplemented with gentamicin (50µg/ml, Gibco®) for the first 7 days in culture.

3ml of medium was removed and replaced with fresh SN culture medium every 3-4 days. The majority of embryonic mouse derived (eM) neurospheres were expected to develop within 10-14 days of the cells being placed in culture (Almond*, et al* 2007). Neurospheres were passaged using a further combination of trypsin digestion and mechanical dissociation. Neurospheres were washed with PBS, centrifuged at 150g for 5 minutes and then incubated at 37°C with 0.05% (w/v) trypsin for 15 minutes. Mechanical dissociation was then performed as above and 5x10^5 cells placed in fresh 60mm non-adherent dishes (751750, Sterilin, Newport, UK) with a final volume of 6ml of SN medium.

2.3.1.2. Neonatal mouse

To generate neonatal mouse (nM) neurospheres, residual faecal material left within the neonatal mouse colons (prepared in 2.1.2) was removed by gentle pressure using non-toothed forceps. Colons were transferred to a fresh dish containing DMEM (2.1.1) supplemented with gentamycin (10ng/ml), penicillin (100 U/ml) and streptomycin (0.1% w/v) and cut into 1mm x 1mm pieces. These were then placed in a 15ml centrifuge tube with collagenase and dispase (both 1% (w/v), Gibco®) and incubated in a water bath at 37°C for 1h. The sample was then briefly vortexed before centrifugation at 150g for 5 minutes and two PBS washes. Following the final wash the cells were filtered through a 40µm cell strainer (Falcon®, BD Biosciences, UK), centrifuged one further time at 150g, the supernatant removed and the cells resuspended in 1ml SN medium. Cells were then placed in 3cm dishes and cultured as per embryonic samples (2.3.1.1) with the addition of 0.5µg/ml amphotericin B (Fungizone®, Gibco®) for the first week in culture. Neurospheres from 4-week-old adult mice (aMD neurospheres) were generated using the neonatal mouse protocol.

2.3.1.3. Ganglionic-derived human neurospheres

To generate human ganglionic-derived neurospheres from both patients with HSCR and controls the colonic biopsies (2.1.3) were washed twice with PBS (supplemented with gentamicin at 50µg/ml). Mucosa and sub mucosa were removed using the dissecting microscope and the serosal surface was cleaned of any residual blood vessels of mesentery. The residual tissue (longitudinal/circular muscle and myenteric plexus) was cut into 1mm x 1mm pieces before incubating with collagenase/dispase (as per 2.3.1.2).

After the first 1h incubation samples were vortexed briefly and inspected macro and microscopically. The collagenase/dispase step was then repeated until a single cell

suspension had been obtained, with the majority of samples requiring 2-3 cycles. The samples were then processed and cultured as per neonatal samples (2.3.1.2).

2.3.1.4. Aganglionic-derived human neurospheres

Samples used to generate neurospheres from the aganglionic bowel of patients with HSCR were processed using the same protocol as ganglionic specimens to obtain a single cell suspension. The subsequent culture technique was identical except that HS medium was used instead of SN medium unless specifically stated otherwise, as cells were found to grow more consistently under these conditions. Human ganglionic-derived neurospheres used as comparisons to those derived from the aganglionic bowel were cultured under the same conditions as human aganglionic-derived neurospheres.

2.3.2. Colonic smooth muscle cultures

2.3.2.1. Mouse colonic smooth muscle

Colon from adult mice was removed (2.1.2) and pinned onto a pre-prepared dissection dish containing sterile PBS. The colon was opened longitudinally, any residual faecal material removed, and the mucosa and submucosa separated from the underlying muscle and discarded. The remaining muscle was cut into 1mm x 1mm pieces and incubated with collagenase/dispase (see 2.3.1.2) until a single cell suspension was obtained. Cells were counted and 1-2x10^6 cells transferred to adherent 6cm dishes (Nalge Nunc, New York, USA) in a total of 6ml FB medium, supplemented for the first 7 days with 0.5µg/ml amphotericin B and gentamicin (50µg/ml). Confluent dishes were dissociated by a combination of enzymatic digestion (0.05% w/v trypsin for 10mins at 37˚C) and mechanical dissociation and transferred to T75 flasks (Corning). Flasks were passaged every 7 days or when cells had reached confluence.

2.3.2.2. Human colonic smooth muscle

Primary human colonic smooth muscle cultures were obtained from both Hirschsprung's patients (ganglionic and aganglionic colonic biopsies) and controls (see table 2.1). Cultures were established using the same methods as for adult mouse (2.3.2.1).

2.3.3. HEK293T cell culture

The human HEK293T cell line (provided by Dr T McKay, University of Manchester) was used to amplify lentiviral particles (Demaison*, et al.* 2002) (see 2.6). Aliquots of cells were stored in DMSO cell freezing medium (Life Technologies) in liquid nitrogen until needed. Aliquots were defrosted and grown in T75 flasks (Corning) in FB medium until confluent, at which point cells were incubated with 0.05% (w/v) trypsin for 10 minutes at 37˚C before being mechanically dissociated and transferred to a T125 flask (Corning) in FB medium until required.

2.4. Notch Inhibition

2.4.1. Gamma secretase inhibition

The gamma secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Sigma-Aldrich, see 1.1.4**)** was used to inhibit the Notch signalling pathway (Dovey*, et al.* 2001, Sastre*, et al.* 2001). This was dissolved in dimethyl sulfoxide (DMSO) to obtain a 10mM concentration, before adding it to the required culture dish at a final concentration of 20µM. An equal volume of DMSO was applied to control dishes. The length of incubation was determined by the experiment conducted and is given in the appropriate result section (Chapter 4.2)

2.4.2. siRNA inhibition of RBP-Jκ

Although the administration of DAPT is a common technique to inhibit Notch signalling, the membrane complex that it targets (gamma secretase) is known to be implicated in the intramembrane proteolysis of >100 other substrates (Groth*, et al.* 2012). Therefore a more selective approach was taken to specifically target the Notch pathway using siRNA knockdown of the core component of the canonical pathway RBP-Jκ (Fig 1.2).

Neurospheres were dissociated (as per 2.3.1.1) and 1-2x10^4 cells seeded onto fibronectin coated 8-well chamber slides (2.7.2.1, Thermo Scientific, Leicestershire, UK) in a volume of 500µl (SN medium). Cells were allowed to adhere for 3 hours prior to transfection. Transfection was performed according to the manufacturer's instructions using 1.5µl of the transfection reagent (HiPerFect, Qiagen, Manchester, UK) and 10nM of each siRNA oligo (Table 2.2). Cells were fixed at 72hrs with 4% (w/v) paraformaldehyde prior to analysis.

Table 2.2. List of siRNA oligos used

siRNA	Sequence	Manufacturer
hsRBPJ_1	TAGGGAAGCTATGCFAAATTA	Qiagen
hsRBPJ_2	GTGGCTGGAATACAAGTTGAA	Qiagen
hsRBPJ_3	CACGGTATTATAGTACACCTT	Qiagen
All Stars Human cell death control	Proprietary	Qiagen
All Stars negative control	Proprietary	Qiagen

Table 2.2. siRNA oligos were a standard panel targeting independent regions of RBP-Jκ produced by Qiagen

2.5. Transplantation Assays

To determine the functional potential of human aganglionic-derived neurospheres we transplanted human neurospheres into an embryonic, aganglionic bowel model and measured the effect on bowel contractility (Lindley*, et al* 2008).

2.5.1. Bowel explant culture

Time-mated CD1 female mice were culled at 11.5dpc and the distal hindgut harvested from the embryos as detailed in shown in Fig. 2.1. Aganglionic explants were either cultured alone as negative a control or transplanted by placing a single aganglionic-derived neurosphere at its proximal end (Fig. 2.2). Ganglionic explants were cultured without transplantation as a positive control. Up to a maximum of four hindgut samples were maintained on each insert and were maintained in culture (37°C, 5% CO_2) for a total of 8 days before assessment.

Figure 2.2 Culture conditions for bowel explants.

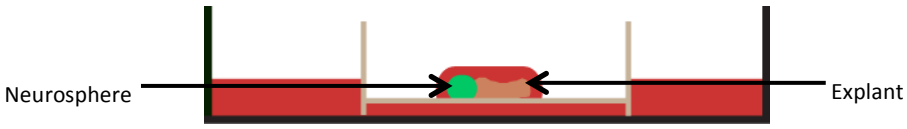


Figure 2.2. Distal hindgut explants were cultured on a semi-permeable 0.4µm Millicell® insert (Merck Millipore, Hertfordshire, UK) placed within a 6cm culture dish containing 5ml of TC medium. At 24h 40µl of TC medium was pipetted on top of the explant. Culture medium was changed every 2 days.

2.5.2. Assessment of bowel contractility

The 6cm dishes containing the Millicell® inserts were transferred directly to a Nikon TMS-F inverted microscope (Nikon UK, Surrey, UK) for analysis. The microscope stage was maintained at 37°C using a Techne MS2 hotplate (Bibby Scientific, Staffordshire, UK) and 35mm thick custom made polystyrene hood. Bowel contractions were recorded using a KP143 video camera (Hitachi Europe, Berkshire, UK) and Piccolo video card (Euresys, Angleur Belgium). Diamtrak software (Prof Nield, Flinders University, Adelaide, Australia) was used to measure both the frequency and amplitude of contractions (Lindley*, et al* 2008). 5-minute recordings were taken from each explant and individual contractions defined as a ≥ 5% displacement of the bowel wall, measured at the point of maximal displacement. To prevent a decrease in contractility due to cooling, a maximum of 2 recordings were made with each explant before returning to the incubator for ≥30minutes.

2.6. Lentiviral transfection

To enable live cell tracking lentiviral transfection techniques were used to induce the expression of green fluorescent protein (GFP) in cells.

2.6.1. Plasmid cloning

A combination of three plasmids was used to produce a GFP expressing lentivirus (Table 2.3). Plasmids were transformed using One Shot® TOP10 chemically competent *Escherichia coli* according to the manufacturer's instruction (1µg of plasmid, Life Technologies). Bacteria were plated onto Lysogeny broth (LB) agar coated dishes in the presence of 100µg/ml ampicillin (Sigma-Aldrich), successful colonies were selected and plasmid purification performed using a Plasmid Plus Maxi-prep (Qiagen) following the manufacturer's instructions.

Table 2.3. List of plasmids used

Plasmid	Size	Role
pMDG.2	5824bp	Encodes the envelope protein (Vesicular-stomatitis-cirus glycoprotein) that help stabilise the vector and improve the ability to infect target cells
pCMVΔ8.64	13463bp	Encodes the packaging plasmid which includes the Group specific antigen (GAG) gene (capsid protein) and Pol gene(reverse transcriptase/integrase activity)
pHR-SFFV-eGFP	9662bp	Contains the restriction sites and reporting (GFP) gene to promote GFP expression in the host cell. SFFV = spleen focus forming promoter.

(All provided by Dr T McKay, University of Manchester)

2.6.2. HEK293T transfection

1.2-1.5x10^7 HEK293T cells were seeded into a fresh T125flask and cultured in FB medium overnight at 37°C in the presence of 5%CO_2. 5ml of DMEM (4.5g glucose/100ml, 25mM, Life Technologies) was supplemented with 250µl of 1M $CaCl_2$ before bringing the pH to 7.1 with 1M HCl and passing it through a 0.2µm sterile filter (Whatman™ International Ltd, Kent, UK). The plasmids were then added to this solution in the following amounts: 50µg pHR-SFFV-EGFP, 32.5µg pCMVΔ8.64 and 17.5µg pMDG.2. After maintaining the plasmid

solution for 20minutes at room temperature 20ml of FB medium (pH adjusted to 7.9) was added to make a final volume of 25ml. The HEK293T medium was then removed, replaced with the plasmid solution and the flask returned to the incubator for 48h (replacing the medium with 20ml FB medium again at 24h). At the end of the final incubation period the culture medium was removed, filtered to remove cell debris (0.2µm) before transferring to a Vivaspin® 20 separation tubes (Sartorius Stedim, Surrey, UK) and spun down for 60minutes at 1500g (4°C). The resulting concentrated viral suspension was aliquoted into cyrovials (200µl, Corning) and stored at -80°C until required.

2.6.3. Viral transductions

Lentiviral transduction was performed on mature, passage 3 human ganglionic-derived neurospheres cultured in SN medium. All neurospheres from a single 60mm dish were selected and incubated with 0.05% (w/v) Trypsin for 15 minutes, after which a single cell suspension was produced with gentle mechanical dissociation. Cells were centrifuged at 150g for 5min and seeded into a fresh 60mm non-adherent dish in SN medium mixed with 1 aliquot of viral suspension (2.6.2). Cells were maintained under standard conditions, replacing the SN medium at 24h, GFP expression was typically >90% and GFP+ve neurospheres developed by day 10 to 14.

Figure 2.3 GFP Transfection of human ganglionic derived neurospheres

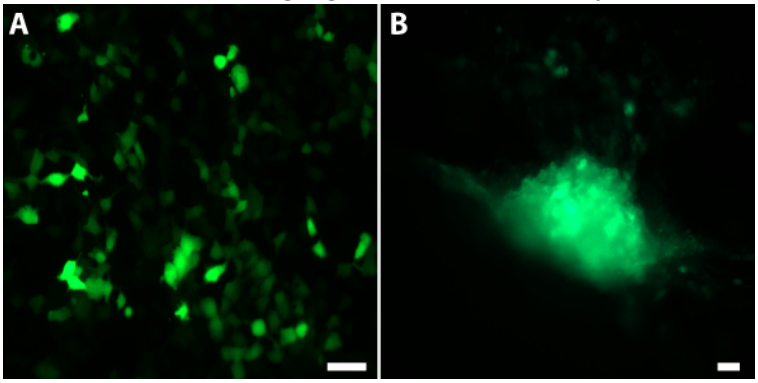


Figure 2.3. A dissociated cells 48h after transfection. **B** GFP neurospheres at 2 weeks demonstrating GFP expression in the majority of cells.

2.7. Immunohistochemistry

2.7.1. Tissue section preparation

2.7.1.1. Full thickness colonic frozen sections

Full thickness sections were taken from specimens of human colon, mouse colon and colonic smooth muscle models (CSMMs) with a maximum size of 3x3x3mm. Specimens were fixed in 4% (w/v) paraformaldehyde for 1-2h at room temperature, washed twice in PBS before placing in 20% (w/v) sucrose overnight at 4°C for cryoprotection. The following morning specimens were transferred into Peel-A-Way® moulds (Polysciences Europe, Eppelheim, Germany), embedded in 500µl of Shandon Cyromatrix (Thermo Scientific) and stored at -80°C until sectioning.

Sections were cut at 7µm (unless otherwise stated) using a MX35 microtome blade and HM505N cryostat at -22˚C before mounting onto Superfrost® Plus microscope slides (all Thermo Scientific). Slides were stored at -80°C until required.

2.7.1.2. Neurosphere frozen sections

Frozen sections of neurospheres were fixed and transferred into embedding moulds using the same protocol. To ensure that neurospheres were correctly orientated at the bottom of each mould, moulds were centrifuged at 150g for 3 minutes before transferring to -80°C. Sections were cut as per 2.7.1.1.

2.7.1.3. Paraffin sections

Paraffin sections were taken from CSMMs to allow more detailed analysis of the architecture within tissue sections. Specimens were fixed with 4% (w/v) paraformaldehyde as per 2.7.1.1 before dehydrating and embedding in paraffin (see table 2.4).

Table 2.4. Embedding steps for paraffin sections

Time	Solution	Temperature
15mins	50% ethanol	Room Temp
15mins	75%	Room Temp
15mins	96%	Room Temp
15mins	100% ethanol	Room Temp
30mins	50% isopropanol 50% paraffin	60°C
30mins	100% paraffin wax	60°C

Blocks were allowed to set at room temperature overnight and stored at 4°C until required. Ribbons were cut from blocks at 4-7µm using S35 microtome blades (Feather®, Osaka, Japan) and a Shandon Finesse 325 microtome before mounting onto Superfrost® Plus microscope slides using a Raymond Lamb section mounting bath (All Thermo Scientific). Once mounted slides were kept at 4°C until required.

2.7.2. Dissociated neurosphere preparations

To allow determination of expression at single cell level neurospheres were dissociated (as per 2.3.1.1) and either seeded onto coated glass chamber slides and maintained in culture or immediately spun down onto slides to allow a more accurate assessment of expression in a freshly dissociated neurospheres.

2.7.2.1. Chamber slide preparation

To improve cell adherence glass 8-well chamber slides (Thermo Scientific) were coated under aseptic conditions with Fibronectin (Merck Millipore) or Poly-D-Lysine and Laminin (both Sigma-Aldrich) to promote neuronal differentiation:

Fibronectin coating: Each chamber was coated with 1µg of Fibronectin in 200µl PBS. This was left for 1h at 37°C before washing once with sterile PBS and using immediately.

Poly-D-Lysine & Laminin Coating: Chambers were first coated with 10µg/cm^2 of Poly-D-Lysine (Sigma-Aldrich), in 200µl PBS, for 8h at room temperature. Slides were washed twice with distilled H_2O and then coated with 1µg/cm^2 of Laminin in 200µl PBS. Slides were left for a further 8h at room temperature to allow full coverage before washing twice with distilled H_2O and allowing to air dry. Slides were stored at 4°C until required.

2.7.2.2. Cell centrifugation for single cell analysis

To minimise changes in expression following dissociation of neurospheres, freshly dissociated cells were immediately spun down onto slides using a Shandon Cytospin3 (Thermo Scientific). Neurospheres were incubated with 0.05% (w/v) Trypsin for 15mins at 37°C before adding 2ml of the medium the neurospheres had previously been cultured in to stop any further digestion. Samples were centrifuged at 150g for 5min and resuspended in 1ml of PBS. The cell concentration was determined using a haemocytometer and the

concentrated adjusted to $1x10^4$ cells/ml with PBS. 500µl of this suspension was added to each cytospin funnel and spun onto Superfrost® Plus slides at 60g for 10minutes. Slides were fixed with 4% (w/v) paraformaldehyde and processed immediately.

2.7.3. Staining protocols

2.7.3.1. Haematoxylin and eosin (H&E)

Slides were stained with H&E using the steps in table 2.5. For frozen sections steps 1 and 2 were omitted. Following the final Histoclear step slides were air-dried and mounted with ProLong® Gold antifade reagent (Life Technologies), and left to cure overnight at room temperature.

Table 2.5. Steps for H&E staining of paraffin sections

Step	Solution	Time
1	Histoclear I / II (Thermo Scientific)	5mins each
2	100% / 96% / 75% / 50% Ethanol	2mins each
3	Haematoxylin	10mins
4	Tap water	Briefly
5	0.1% HCl	10secs
6	Warm tap water	5mins
7	Eosin	2mins
8	Tap water	Briefly
9	50% / 75% / 96% / 100% ethanol	2mins each
10	Histoclear II / I	2mins each

Table 2.5 The same protocol was used for frozen sections omitting steps 1 and 2.

2.7.3.2. Standard immunofluorescence

To prepare for staining slides were washed with PBS (3X) before regions of interest to stain were marked out with a hydrophobic PAP marker pen (DAKO, Cambridgeshire, UK). Unfixed slides were then incubated with 4% (w/v) paraformaldehyde for 10min at room temperature before a further 3 PBS washes followed by the immunofluorescence blocking

buffer (IBB) (see 2.9.1) for at least 1h at room temperature to block and permeabilise the cells. Primary antibodies were diluted to the required concentration in PGN, applied and incubated overnight at 4°C.

The following day, slides were washed with PBS (X3) before incubating with the appropriate secondary antibody (in PGN) for 1h at room temperature. Following a further 3 washes with PBS cells were mounted using ProLong® Gold antifade reagent with DAPI (Life Technologies) and coverslips (Thermo scientific). Slides were cured at room temperature before analysis.

2.7.3.3. Assessment of proliferation

To identify proliferating cells within a cultured population either bromo-deoxyuridine (BrDU, Beckton Dickinson) or a 5-ethynyl-2'-deoxyuridine (EdU) ClickIT™ Imaging kit (Life Technologies) was used. BrDU and EdU are nucleoside analogues of thymidine that are incorporated into DNA during the S phase of the cell cycle (Salic*, et al.* 2008).

BrDU was added to cell cultures at a final concentration of 10µM for the desired length of time specific to each experiment. Subsequently, frozen sections would be prepared as described in 2.7.1.1/2 except that prior to the addition of Triton, 4M hydrochloric acid was applied to each slide for 15 mins in order to denature the DNA and expose the BrDU group. In contrast, when using EdU the incorporated EdU can then be detected using an AlexaFluor®594 conjugated azide, which binds covalently to the alkyne group of EdU in a copper-catalysed reaction. This has the advantage over BrDU as it does not require a DNA denaturation step and allows co-stained for other antibodies.

Cells were incubated with 20mM EdU for the desired length of time according to the individual experiment design. Samples were then fixed and permeabilised using the standard protocol (2.6.3.2). Detection of incorporated EdU was detected using according to the ClickIT™ manufacturer's instructions.

2.7.4. Primary antibodies

Table 2.6. Primary antibodies used in immunofluorescence assays.

Antibody	Host	Supplier	Dilution
Calretinin (ab702)	Rabbit Polyclonal IgG	Abcam[1]	1:25
Caspase 3 (ab2171)	Mouse monoclonal IgG2a	Abcam	1:50
ChAT (ab68779)	Rabbit Polyclonal IgG	Abcam	1:200
GFAP (Z0334)	Rabbit Polyclonal IgG	Dako	1:250
GFAP (G3893)	Mouse Monoclonal IgG1	Sigma-Aldrich	1:250
Jagged 1 (ab7771)	Rabbit Polyclonal IgG	Abcam	1:500
Ki67 (ab15580)	Rabbit Polyclonal IgG	Abcam	1:100
PGP 9.5 (ab8189)	Mouse Monoclonal IgG2a	Abcam	1:100
P75 (ab3125)	Mouse Monoclonal IgG1	Abcam	1:1000
P75 (ab8874)	Rabbit Polyclonal IgG	Abcam	1:1000
P75-PerCP/Cy5.5 (345112)	Mouse monoclonal $IgG1_K$	Biolegend[2]	1:500
nNOS (ab63602)	Rabbit Polyclonal IgG	Abcam	1:500
Notch 1 (ab5267)	Rabbit Polyclonal IgG	Abcam	1:200
Notch 1 NICD (ab52301)	Rabbit Polyclonal IgG	Abcam	1:100
Notch 2 (ab8926)	Rabbit Polyclonal IgG	Abcam	1:500
Notch 3 (ab23426)	Rabbit Polyclonal IgG	Abcam	1:500
Notch 4 (N5163)	Rabbit Polyclonal IgG	Sigma-Aldrich	1:500
SMA (ab7817)	Mouse Monoclonal IgG2a	Abcam	1:200
S100 (ab868)	Rabbit Polyclonal IgG	Abcam	1:500
Tuj (MMS-435P)	Mouse Monoclonal IgG2a	Convance[3]	1:500
VIP (ab43841)	**Rabbit Polyclonal IgG**	**Abcam**	**1:1000**

Table 2.6. Supplier contact details: [1]Abcam, Cambridgeshire, UK. [2]Biolegend UK, London, UK. [3]Convance UK, Berkshire, UK.

2.7.5. Secondary antibodies

Table 2.7. Secondary antibodies used in immunofluorescence assays

Antibody	Host	Supplier	Dilution
Alexa Fluor 594 - Anti-rabbit	Goat IgG	Life Technologies	1:1000
Alexa Fluor 488 - Anti-rabbit	Goat IgG	Life Technologies	1:1000
Alexa Fluor 594 - Anti-mouse	Goat IgG	Life Technologies	1:1000
Alexa Fluor 488 - Anti-mouse	Goat IgG	Life Technologies	1:1000

2.8. Molecular biology

2.8.1. RNA extraction

RNA was extracted from both neurospheres and freshly isolated colonic tissue.

2.8.1.1. Neurospheres

50-100 spheres were individually isolated from a culture dish using a 100µl pipette and placed in sterile PBS. This suspension was centrifuged, at 100g for 5 minutes, resuspended in 1ml TRIzol® (Life Technologies) to lyse the cells and transferred into a 1.5ml microcentrifuge tube (Starlab, Hertfordshire, UK). After 5 min 200µl chloroform (Sigma-Aldrich) was added and the suspension agitated vigorously. The sample was then centrifuged at 12 000g for 15 minutes (4°C). The resultant upper aqueous layer containing RNA was then transferred to a fresh microcentrifuge tube and an equal volume of isopropanol (Sigma-Aldrich) and 1µg of glycogen (Life Technologies) added. Samples were then kept at -20°C overnight.

2.8.1.2. Colonic tissue

Samples were cut into pieces (<1x1x1mm), transferred to a 1.5ml microcentrifuge tube and 500µl of TRIzol® added. The sample was homogenised before adding a further 500µl of TRIzol® and continuing the protocol as for neurospheres.

Samples were stored at -20°C for at least 12h, to increase the RNA yield, before centrifuging at 12 000g for 10 minutes (4°C). The supernatant was discarded and RNA/glycogen pellet resuspended in 75% (v/v) ethanol (Sigma-Aldrich) in nuclease free water (Gibco®). Samples were spun down a further time at 4400g (5mins, 4°C), supernatants discarded and pellets briefly air-dried. After resuspending in 20 µl of nuclease free water the RNA concentration was determined using a Biophotometer spectrophotometer (Eppendorf UK, Hertfordshire, UK) and samples diluted to 200ng/µl in 8µl aliquots.

2.8.2. cDNA synthesis

DNase treatment was performed prior to cDNA synthesis to prevent contamination with residual genomic DNA. To achieve this, 1µl each of DNase and RQ1Buffer (both Promega, Hampshire, UK) were added to each 8µl RNA aliquot and the reaction incubated at 37°C for 30 minutes using a thermal cycler (2720, Applied Biosystems, Life Technologies). The reaction was terminated by the addition of 1µl of Stop Buffer (Promega) and a 15min incubation step at 60°C.

To synthesise cDNA, 1µl (100ng/µl) of random hexamers (Abgene, Thermo Scientific) and 1µl of a 10mM dNTPs mix (Bioline Ltd, London, UK) were added to the DNase treated RNA and incubated at 4°C for 3minutes. 1µl of 200U/µl Superscript III reverse transcriptase (Promega), 4µl of 5X First strand buffer and 1µl of 100mM dithiothreitol (both Bioline) were then added and the reaction mixture incubated at: 25°C for 5 minutes, 50°C for 60 minutes and 70°C for 15 minutes in the thermal cycler. The resultant cDNA product was then stored at -20°C until required.

2.8.3. qRT-PCR reaction

A Rotor Gene SYBR Green RT-PCR kit (Qiagen) and Rotor-Gene RG-3000 Thermal cycler (Corbett Research, Qiagen, UK) were used for the final step of the qRT-PCR reaction. The reaction mix was prepared on ice (as per table 2.8) to a final volume of 25µl. The Rotor Gene cycler was programmed as per the manufacturer's fast two-step RT-PCR protocol for a total of 40 cycles. The qPCR reaction step for RBP_jK was kindly performed by Hannan Al-Lamee.

Table 2.8. Reaction set up for qRT-PCR

Component	Volume	Final concentration
2x Rotor-Gene SYBR Green PCR master mix	12.5µl	1x
10x QuantiTect Primer	2.5µ	1x
Template cDNA	1µl	≤100ng/reaction
RNase-free water	9µl	-

2.8.4. Primers

Table 2.8. List of primers used.

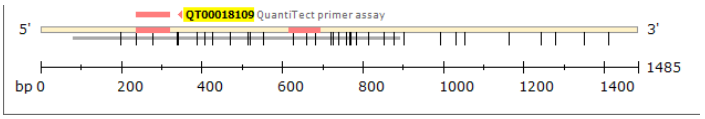
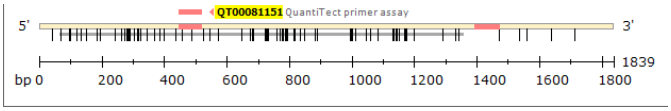
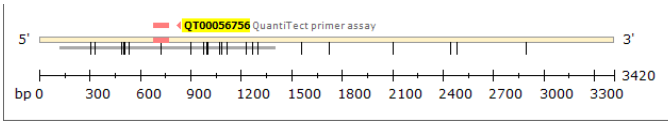
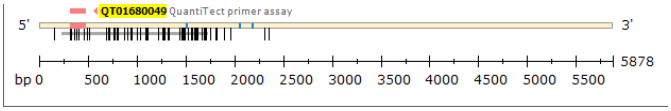
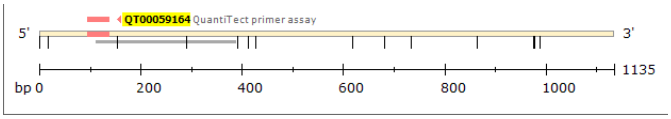
Gene	Details	Amplicon length
ß-actin	F: 5'-CGTTGACATCCGTAAAGACC-3' R: 5'-CAGGAGGAGCAATGATCTTGA-3'	143 bp
Calbindin2 NM_00174) 1485 bp	QuantiTect® kit primer assay QT00018109 (Forward/reverse mix) 	107 bp
GFAP NM_001131019 96 bp	QuantiTect® kit primer assay QT00081151 (Forward/reverse mix) 	96 bp
P75 NM_002507 3420 bp	QuantiTect® kit primer assay QT00056756 (Forward/reverse mix) 	118 bp
RBP$_j$K NM_005349 5878 bp	QuantiTect® kit primer QT01680049 (Forward/reverse mix) 	185 bp
S100B NM_006272 1135 bp	QuantiTect® kit primer assay QT00056756 (Forward/reverse mix) 	64 bp

Table 2.8. All primers and gene maps supplied by Qiagen.

2.9. Image acquisition and analysis

2.9.1. Microscopy

Light microscopy was used for the majority of image acquisition and analysis either with a Leica DM IRB Inverted microscope or Leica DMRB Upright microscope using a Leica DFC420C camera (All Leica Microsystems). A Leica M165FC dissection microscope and Leica DFC425C camera were used for live imaging of the CSMM and transplant experiments. Confocal images were taken with a Leica TCS SPE microscope with integrated camera.

2.9.2. Image analysis software

Adobe Photoshop (Adobe Inc., California, USA) was used for merging, resizing and cropping. Adobe Illustrator was used to assemble the Figures. Image J (National Institutes of Health, USA) was used for automated cell counting.

2.10. Smooth Muscle Physiology

Smooth muscle testing was performed with the assistance of Dr Rachel Floyd, post-doctoral fellow, Department of Cellular and Molecular Physiology, University of Liverpool.

Constructs were cut into 1cm lengths and buffered physiological saline (154mM NaCl, 5.6mM KCl, 1.2mM $MgSO_4$, 2mM $CaCl_2$, 8mM glucose and 10.9mM HEPES at pH 7.4). Each strip was loaded between a fixed stainless steel hook and a second hook attached to a force transducer (Fort10g, World Precision Instruments, Hertfordshire, UK) in a custom-made Perspex tissue bath. Excess slack was taken up but no stretch applied, so as to not activate stretch channels or rupture cells, causing aberrant contractions.

A 4 channel peristaltic pump (Gilson, Wisconsin, USA) was used to perfuse the samples with either physiological saline, 120mM KCl (composition: 39.6mM NaCl, 120mM KCl, 1.2mM $MgSO_4$, 2mM $CaCl_2$, 8mM glucose and 10.9mM HEPES) or 100µm carbachol (in calcium free physiological saline with 2mM EGTA). All chemicals obtained from Sigma, UK.

For each run, samples were superfused with buffered physiological saline (7.8ml/ml at 30˚C) for 5 minutes before beginning testing.

KCl Testing: Samples were perfused with 120mM KCl for 5 minutes before washing with buffered physiological saline for 10 minutes.

Carbachol Testing: Samples were perfused with 100µM Carbachol for 2 minutes before washing with buffered physiological saline for a further 10 minutes.

Data was acquired using a LAB-TRAX-4 sata acquisition system coupled with a Transbridge Transducer Amplifier (SYS-TBM4M, World Precision Instruments). Data analysis was performed using OriginPro 8.5 software (Origin Lab, Massachusetts, USA).

2.11. Statistical Analysis

Non-parametric continuous data were analysed using Mann-U Whitney, a two-tailed t test was used for parametric data and Fisher's exact test for categorical data. A 1 -way ANOVA analysis with Tukeys correction was used for parametric grouped data and a Krustal-Wallis test for non parametric. p <0.05 was taken as significant. All analyses were performed using GraphPad Prism Version 5 (GraphPad Software Corp., California, USA).

Chapter 3: Cell Proliferation within Mouse and Human Neurospheres

3.1. Introduction

3.1.1. Background

Defects during the initial colonisation of the gut by cells originating in the vagal neural crest are thought to be key to the development of neurocristopathies. The downstream pressure created by the rapidly proliferating neural crest wave-front is thought to play a significant role in this process (Young*, et al* 2004) (see 1.1.1). Furthermore, previous studies have shown that the small number of cells at this wave-front have the capacity to colonise the entire distal colon during embryonic development (Sidebotham*, et al* 2002). Such embryonic cells and also cells with similar properties have been isolated from the post-natal human colon and have then been maintained in-vitro in the form of neurospheres with the goal of using them in future transplantation therapies (Rauch*, et al* 2006, Almond*, et al* 2007, Lindley*, et al* 2008, Metzger*, et al* 2009, Hotta*, et al* 2013). Current studies use both neurospheres derived from embryonic mouse and postnatal human sources, however there has been little work to directly compare the behaviour of these cells within the microenvironment of the neurosphere in order to determine to what extent the behaviour of cells derived from each source is comparable.

3.1.2. Attribution of work in this chapter

The results in sections 3.3.1 and 3.3.2 include work carried out by the author and Dr S Theocharatos, a previous PhD student within the lab. Part of the work in this chapter has been published in Plos One (Theocharatos*, et al.* 2013)

3.1.3. Aims

The current literature includes studies using neurospheres derived from both embryonic mouse and postnatal human sources to investigate the possibility of using these cells for transplantation-based therapies. Therefore, the aim of this chapter is to compare the proliferative behaviour of ENSPC derived from both sources in the microenvironment of the neurosphere, and also to determine if any differences were due to differing ages (embryonic vs. postnatal, or species (mouse vs. human).

3.2. Results

3.2.1. Neurosphere formation is influenced by cell density

Before focusing on the behaviour of cells within the microenvironment of the neurosphere, initial experiments looked at the process of neurosphere formation itself. Questions remain regarding whether neurospheres form from single 'stem cell' or arise from cells aggregating together. On observing the formation of neurospheres four distinct phases could be identified: Initially, freshly isolated cells were seen to adhere to the bottom of culture dishes, cells would undergo a period of proliferation before bunching together and then finally 'budding-off' free floating neurospheres (Fig 3.1).

Figure 3.1. Formation of human ENS neurospheres

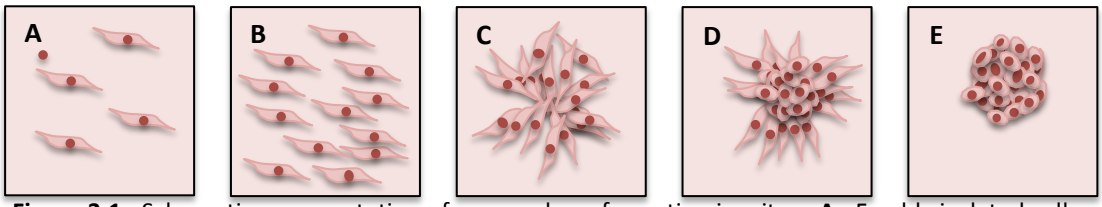


Figure 3.1. Schematic representation of neurosphere formation in–vitro. **A:** Freshly isolated cells adhere to the bottom of culture dishes. **B:** Cells then proliferate before bunching together. **C:** Neurospheres begin to form. **D**: Neurospheres eventually 'bud-off'. **E**:. neurospheres then float freely

It was found that increasing the density at which cells were seeded after neurosphere dissociation could decrease the time taken for neurospheres to begin to form. After 1 week in culture cells seeded at 50K/ 3cm dish were found to have become almost fully confluent (Fig 3.2A), a marked increase in cell confluence could be seen in dishes seeded at 100K/dish (Fig 3.2B). However there was no evidence of neurosphere formation. This changed in dishes seeded with 250K cells, were cells could be seen to bunch together which is the first phase before neurospheres form and 'bud' off (Figure 3.2C). Doubling the cell density again led to the formation of free floating neurospheres in the same time period demonstrating that increasing cell density decreases the time taken for neurosphere formation (Figure 3.2D).

Figure 3.2. Rate of neurosphere formation is influenced by cell density

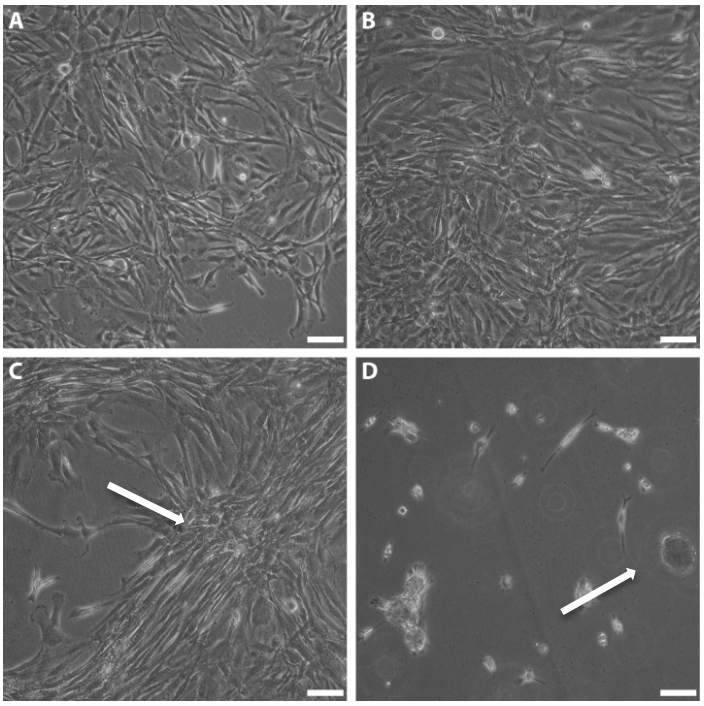


Figure 3.2. Primary human neurospheres were dissociated, counted, and then seeded into 3cm dishes at cells densities of 50K cells/dish (**A**), 100K cells (**B**), 250K cells (**C**) and 500K cells (**D**). Dishes were kept in culture under standard conditions for 7 days prior to analysis. The rate of neurosphere formation increased with higher cell densities. Cells can be seen to bunch together, the first stage of neurosphere formation, in **C** (arrow). Free-floating formed neurospheres were identified at 7 days only in dishes seeded with 500K cells (D, arrow). Scale bars = 50μm.

3.2.2. Expression and localisation of typical ENS markers

Neurospheres were generated from both embryonic mouse and post-natal human sources as previously described (see 2.1). Established, third generation neurospheres were selected from both sources and frozen sections prepared for immunostaining for typical markers expressed within the ENS (Figure 3.3).

The neural crest marker P75 was found to be expressed by the majority of cells within both human and mouse neurospheres. Expression was seen throughout neurospheres, with no significant difference discernable between either species (Figure 3.3A-B). Similarly, expression of the glial marker GFAP was widespread with no specific localisation identified (Figure 3.3C-D). Expression of the mature glial marker S100 was more limited, seen only in a minority of cells which were predominantly located away from the peripheries of the neurosphere (Figure 3.3E-F). In contrast, the early neuronal marker ß-tubulin III (Tuj) was found to be expressed predominantly by cells at the periphery of both mouse and human neurospheres (Figure 3.3G-H).

Figure 3.3. Expression of typical ENS markers in mouse and human neurospheres

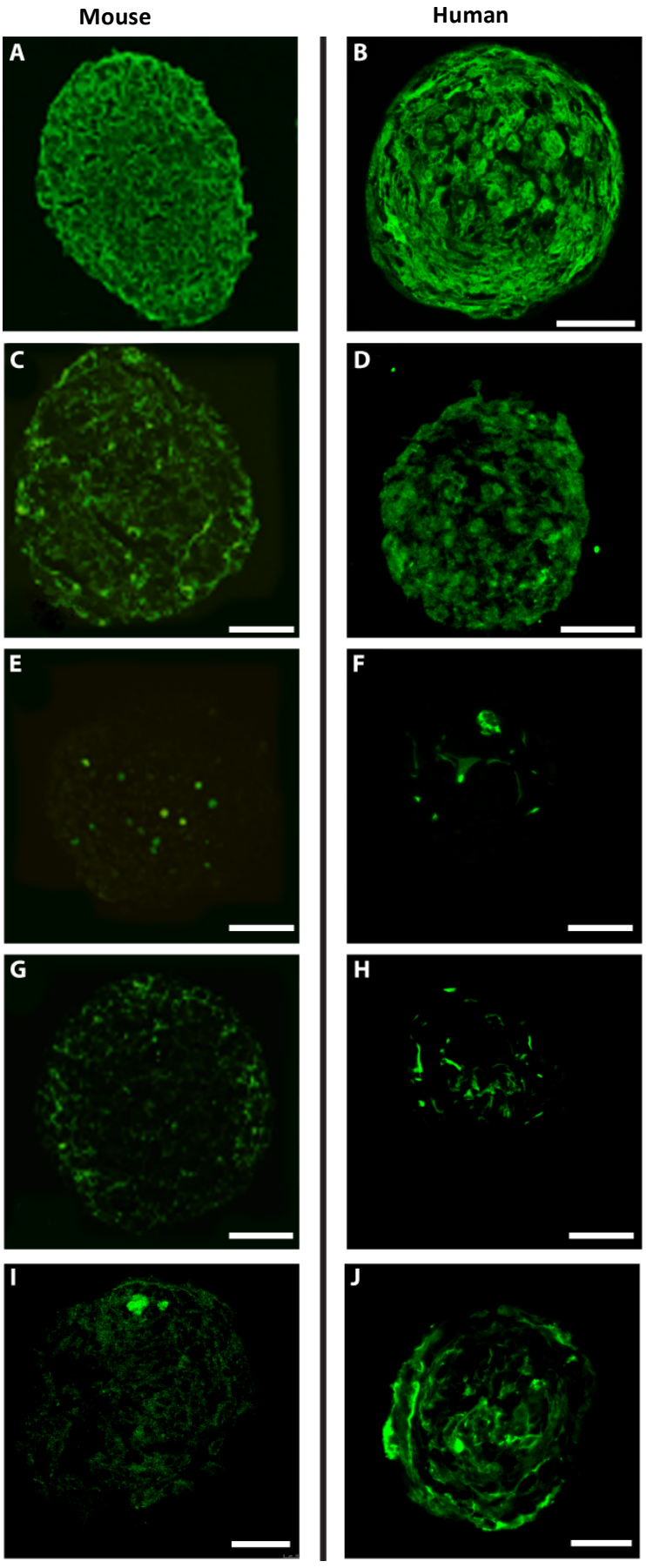


Figure 3.3. 7µm PFA fixed frozen sections taken from 3rd generation mouse (A,C,E,G) and human (B,D,F,H) neurospheres. Sections used for analysis were taken through the centre of each neurosphere, defined as the sections with the maximal diameter taken from each individual neurosphere. Antibodies were used against the neural crest marker P75, the immature glial marker GFAP, mature glial marker S100, early neuronal marker Tuj and smooth muscle actin (Taken from 1st generation neurospheres). **A-B:** P75 expression is expressed throughout both mouse (**A**) and human (**B**) neurospheres. **C-D:** Similarly, GFAP expression has a widespread distribution in sections taken from both mouse (**C**) and human (**D**) neurospheres. **E-F:** S100 is only expressed by a minority of cells in mouse (**E**) and human (**F**) neurospheres. **G-H:** The neuronal marker Tuj has a predominantly peripheral distribution in both mouse (**G**) and human (**H**) neurospheres. **I-J: The smooth muscle marker SMA can be seen throughout early (primary) neurospheres. This expression drops markedly in later generations (data not shown).** Scale bars = 50µm

3.2.3. Localisation of proliferation in embryonic mouse neurospheres is predominantly peripheral

Having identified a difference in the localisation of cells expressing glial and neuronal markers attention was turned to proliferation. The use of thymidine analogues as a proxy for proliferation is well documented in the literature (Salic*, et al* 2008). Initial experiments in this chapter use the traditional analogue BrdU, however the need to treat the cells with hydrochloric acid to detect the incorporated BrdU limited the ability to counterstain cells for additional markers. Therefore, later experiments utilised the alternative analogue EdU, eliminating the need for acid treatment. Studies have demonstrated that there is no difference in the detection of cells entering S phase between EdU and BrdU in skeletal tissues (Salic*, et al* 2008, Mead*, et al.* 2014), and although not included in this thesis, work performed by colleagues within the lab found no difference using either analogue in ENS cells (Theocharatos 2011).

Initial experiments in embryonic mouse neurospheres used the incorporation of a detectable thymidine analogue (BrdU) as a proxy for proliferation (Figure 3.4). Following a 6h incubation period with the thymidine analogue the most striking observation was that almost all cells had been in S-phase at some point during the incubation period, and were located at or very close to the periphery of the neurosphere (figure 3.4A,C). Neurospheres continuously incubated with the analogue for a further 90h showed that the majority of cells appear to have undergone proliferation with positive cells seen throughout the neurosphere (Figure 3.4B).

In contrast, if BrdU was removed from the culture conditions after an initial 6h pulse and the cells analysed after a further 90h, strongly labelled cells were predominantly seen at the centre of the neurosphere, with weakly positive cells still seen towards the peripheries (figure 3.4D). These results are represented graphically in figure 3.4E, using a comparison

Figure 3.4. Localisation of proliferation in mouse neurospheres

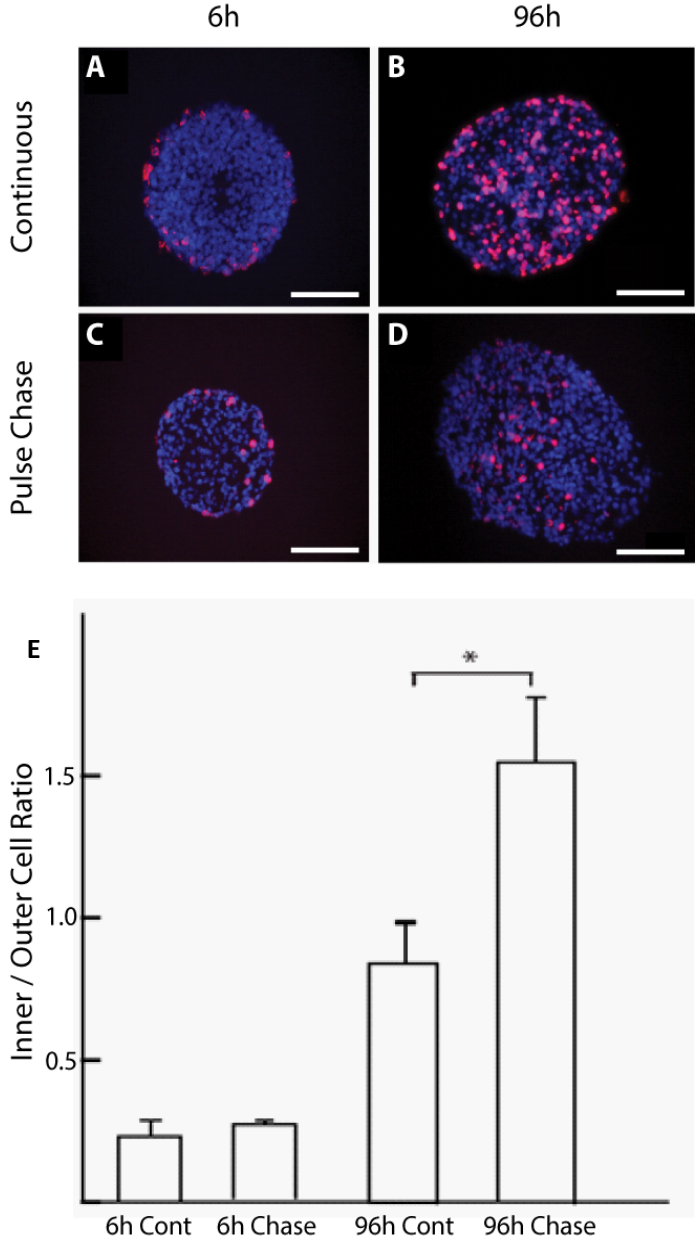


Figure 3.4. Standard 7µm frozen sections through the centre of embryonic mouse neurospheres cultured under standard conditions. **A-B:** Neurospheres were incubated continuously with 10µM BrdU (red) for 6h (**A**) and 96h (**B**). **C,D:** Neurospheres after an initial 1h incubation with BrdU (**C**) and after a further 90h in the absence of BrdU (**D**). Cells are counterstained with DAPI (blue). **E:** Ratio of proliferating cells (incorporating BrdU) between the inner 50% and outer 50% of each section. Scale bars = 50µm. Error bars represent standard error of the mean (SEM) from 5 experiments. * denotes P< 0.01

between the proportion of cells incorporating BrdU in the inner zone (inner 50% of total area) of the neurosphere against the proportion of positive cells found in the outer zone (outer 50%). This demonstrates an almost even distribution between inner and outer zones in neurospheres cultured continuously in the presence of BrdU, with a significant shift towards the proportion of positive cells found in the inner zone under pulse-chase conditions (Figure 3.4E). These results provide evidence that the environment within neurospheres is dynamic, with on going cell movement rather than possessing a fixed architecture.

3.2.4. Following proliferation cells are predominantly located in the central zone of both mouse and human neurospheres

Having seen the apparent movement of post-mitotic cells from a peripheral to a central location within neurospheres the experiments were repeated in both mouse and human neurospheres using an alternative thymidine analogue EdU (Figure 3.5).

Figure 3.5. Post-proliferation cells incorporating EdU are found in the central zones of neurospheres

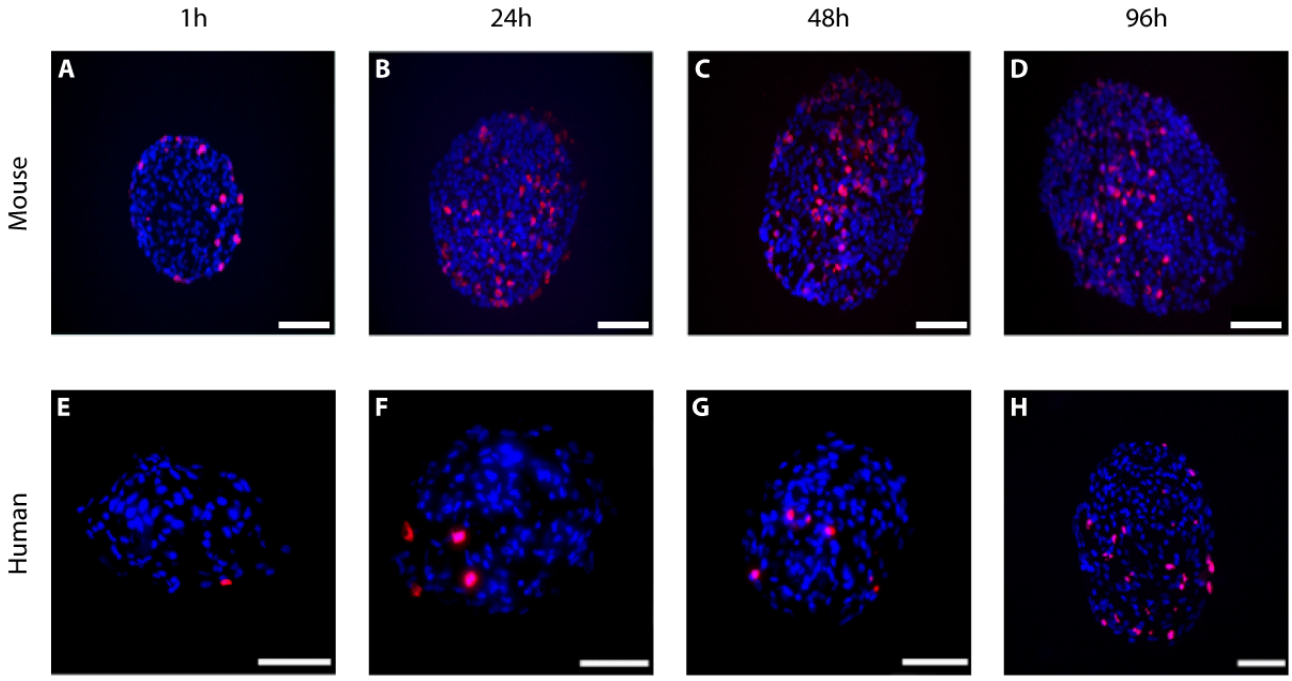


Figure 3.5. 3rd generation embryonic mouse and human neurospheres were incubated in the presence of EdU for 1h, following which it was removed and the neurospheres were cultured under standard conditions for up to 96h. Neurospheres were fixed with 4%PFA and standard 7µm frozen sections prepared through the centre of each neurosphere at set time points. Sections were stained for EdU incorporation (red) and DAPI (blue). **A-D**: Demonstrates the initial peripheral localisation of cells incorporating EdU at 1h in embryonic mouse neurospheres (**A**). Over time cells can be seen throughout the neurosphere, with a predominantly central distribution by 96h (**D**). **E-H:** Pulse-chase experiment repeated with human neurospheres demonstrating a similar pattern of distribution with a marked decrease in the number of cells incorporating EdU. Scale bars = 50µm

There is a marked reduction in the number of cells incorporating EdU in the human neurospheres (Figure 3.5 E-H). However, as seen in the murine neurospheres, over time EdU incorporation was seen in cells in the centre of the neurosphere. As was seen with the BrdU incorporation assays in mouse neurospheres, incorporation of a pulse of Edu in human neurospheres followed a similar pattern, initially occurring predominantly at the periphery of the neurosphere (Figure 3.5 A-D). Over time cells that had incorporated EdU are progressively seen towards the centre of the neurosphere, with the central cells exhibiting a strong signal for EdU incorporation and a much weaker signal in the peripheral cells at later time periods after the pulse.

To eliminate the possibility that the presence of proliferating cells predominantly at the periphery of neurospheres was a penetration artefact due to delayed uptake in central cells, the proliferation marker Ki67 was used on frozen neurosphere sections to demonstrate labelling at the periphery of neurosphere, thereby supporting the hypothesis that proliferation predominantly occurs in these peripheral zones (Figure 3.6). The proportion of cells expressing Ki67 was more than 3 times greater than those incorporating EdU.

Figure 3.6. Proliferation predominantly occurs at the periphery of neurospheres

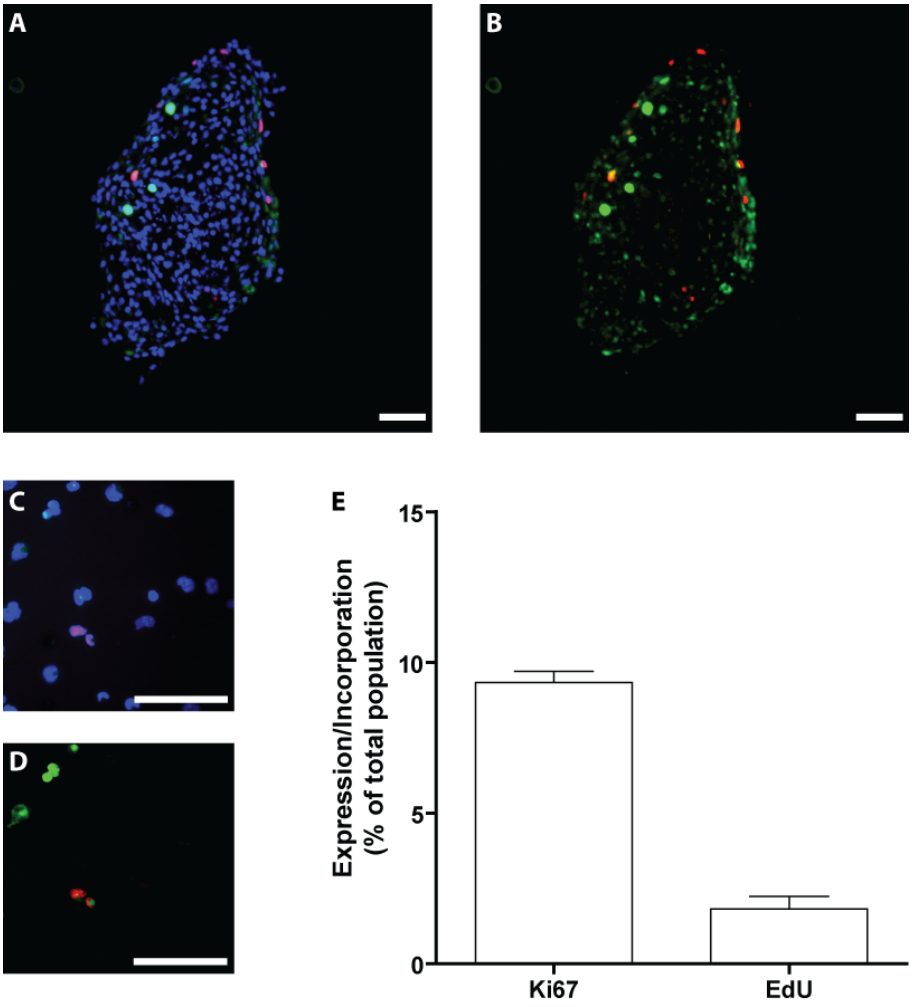


Figure 3.6. Third generation embryonic mouse neurospheres were cultured in the presence of EdU for 1h prior to fixation and preparation of 7µm frozen sections taken through the centre of each neurosphere. Sections were analysed for incorporation of EdU (red) and expression of Ki67(green). Cell nuclei were stained with dapi (blue). **A-B:** Demonstrate the peripheral localisation of both EdU incorporation and Ki67 expression. **D-E:** To enable quantitative analysis neurospheres were dissociated spun down onto slides with a cytospin to determine expression within individual cells. **C**: Expression of Ki67 is significantly higher than incorporation of EdU ($p < 0.0001$). Scale bars = 25µm. Error bars represent standard error of the mean (SEM) from 5 experiments.

3.2.5. Rate of proliferation in neurospheres is determined by cell age

Having seen a difference in the proportion of cells apparently undergoing proliferation in mouse and human neurospheres raises the question as to whether this difference was due to species difference or the difference between embryonic and postnatal cells. Following a 24h incubation with EdU 25% of cells in embryonic mouse neurospheres incorporated EdU, which fell to 20% in neurospheres isolated from day 1 (P1) postnatal mice (Figure 3.7). The most dramatic difference was seen in the behaviour of cells isolated from P1 mice and 28-day old (P28) mice, with a four-fold reduction seen over this time period. Importantly, no significant difference in the proportion of cells incorporating EdU was seen between cells isolated from mature (P28) mice and 3-month old human samples.

Figure 3.7. Differences in the rate of proliferation are primarily dependent on age

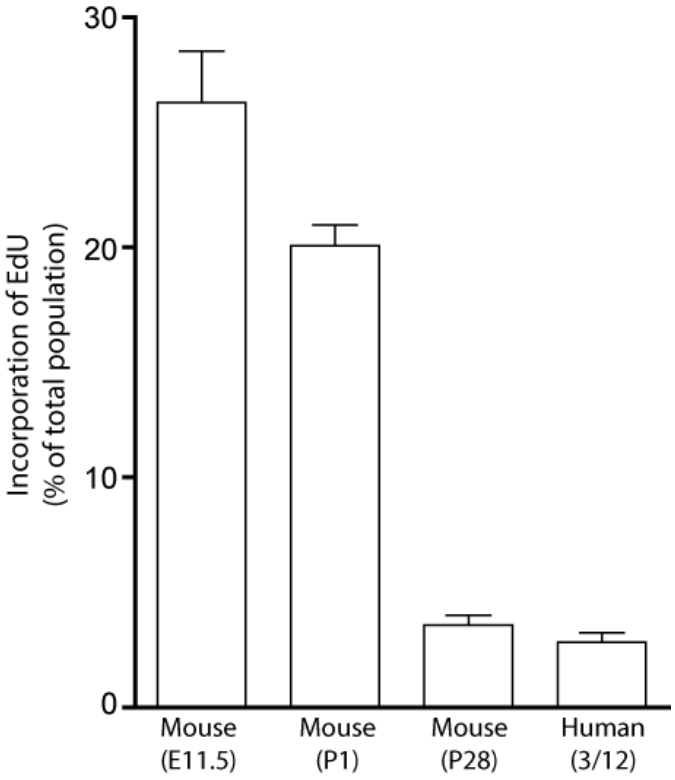


Figure 3.7. Primary neurospheres were isolated from both embryonic (E11.5), 1 day old (P1) and 28 day old (P28) mice and 3-month old human samples. Primary neurospheres were cultured in the presence of EdU for 24h then immediately dissociated and spun down onto slides for fixation and analysis. The proportion of cells incorporating Edu fell significantly between E11.5, P1 and P28 mice ($P < 0.001$). No significant difference was found between P28 mouse and post-natal human neurospheres ($P > 0.05$). Error bars represent standard error of the mean (SEM) from 3 experiments.

3.3. Discussion

The proliferative capacity of NCCs during the development of the ENS is a key factor in the normal colonisation of the bowel. Coupled with the observation that there is an apparent increase in proliferation when ENSPC when cells are isolated from postnatal bowel there is a need to understand firstly what is happening regarding cell behaviour within the artificial microenvironment of the neurosphere niche and secondly whether there are differences in behaviour related to the source of the ENPSC. This chapter details the similarities in both the expression profiles of typical ENS markers between neurospheres derived from embryonic mouse and postnatal human sources and also in the localisation of proliferation within the neurosphere. Furthermore, this chapter confirms that there is a difference in the proliferative behaviour dependent on the development maturity of the source tissue, but not between mouse and postnatal human samples.

3.3.1. Rate of neurosphere formation is influenced by cell density

The majority of studies looking at the formation of ENS derived neurospheres use suspension based cultures (Rauch*, et al* 2006, Almond*, et al* 2007, Metzger*, et al.* 2009), however studies using CNS derived neurospheres have found that under adherent conditions the time to neurosphere formation is significantly reduced.(Konagaya*, et al.* 2011). This, coupled with similar observations within the lab led to the use of adherent culture dishes for the generation of ENS derived neurospheres in this thesis. Using this model, these preliminary results suggest that the density at which ENSPC are seeded significantly reduces the time taken to for neurospheres to form. This could be due to a number of possibilities such as: that by seeding an increased number of cells there will be an increased number of 'true' progenitors within the dish. Therefore, one would expect to see more formed neurospheres. However, what was observed was not a small number of

neurospheres in low density culture with greater numbers seen in the higher density dishes, rather that at 7days formed neurospheres were only seen in the dishes seeded at the highest density. This makes the theory less likely and suggests that it is perhaps the closer/increased interactions between cells that are responsible. Whether the decreased time taken for neurosphere formation is due to an increased rate of proliferation or a change in behaviour is yet to be determined. However, these findings prompted further study in the control of ENSPC behaviour and in particular mechanisms known to be influenced by cell-cell interactions (Chapter 4).

3.3.2. Expression and localisation of typical ENS markers is similar between neurospheres derived from mouse and human sources

Our finding that neurospheres derived from both mouse and human sources typically express common ENS markers is unsurprising and consistent with previous studies (Rauch*, et al* 2006, Lindley*, et al* 2008, Metzger*, et al* 2009). However, it is important that no significant differences in the localisation of each marker could be found between neurospheres derivied from either species.

The two glial markers were used during this study GFAP and S100, both of which are commonly used to label astrocytes in the CNS and have been shown to also label enteric glia(Bondurand*, et al* 2003, Young*, et al.* 2003, Platel*, et al.* 2009). Studies have reported that within the CNS rapidly proliferating 'immature' astrocytes express GFAP and are S100-ve, whereas the expression of S100 is limited to more mature astrocytes (Bannerman*, et al.* 1988, Deloulme*, et al.* 2004, Platel*, et al* 2009). The same pattern is also seen in Schwann cells within the peripheral nervous system with immature schwann cells expressing GFAP and S100 and more mature myelinating cells loosing their expression of GFAP (Mirsky*, et al.* 2008). In this study the more immature marker GFAP was found to be widely expressed,

with perhaps a slight preponderance at the peripheries, whereas the expression of the more mature marker S100 was limited mainly to a more central location. This difference may be linked with the zones of higher proliferation and is discussed further in 3.3.3. The analysis within this study was qualitative only and did not seek to determine the proportion of cells expressing each individual marker, nor did it look at co-expression of markers. Experiments to further elucidate the differential temporal and spatial expression of individual markers was outside the scope of this thesis, but has been performed independently within the lab by S Theocharatos as part of his doctoral studies. His results support the use of GFAP as a marker of immature, proliferating glial cells. He found that the majority of proliferating cells were GFAP+ve, with a subpopulation of cells developing expression of S100 after a period of time (Theocharatos 2011).

3.3.3. Proliferation occurs predominantly at the periphery of neurospheres

Previous studies have demonstrated a similar predominant distribution of proliferating cells at the peripheries of neurospheres derived from both the embryonic rat CNS and embryonic mouse ENS (Campos*, et al.* 2004, Almond*, et al* 2007). However, those studies were limited to short pulses of BrdU and were open to the criticism that such findings were an artefact due to limited penetration of the thymidine analogue. The findings within this chapter provide evidence to support the hypothesis that proliferation within the neurosphere does indeed predominantly occurs in the periphery. Multiple lines of evidence are provided to support this conclusion. Thus, following a chase period positive cells remaining at the periphery are only weakly labelled, suggesting that they had continued to proliferate and have 'diluted' their levels of labelled analogue. Conversely, cells which are located in the central zones of the neurospheres remain highly labelled, which is consistent with these cells that having become post-mitotic and moved away from

the periphery towards the centre of the neurosphere. This is corroborated by analysing the expression of the protein Ki67 which is located in cell nuclei during interphase and would not be affected by inadequate penetration as frozen sections of neurospheres were used for staining (Scholzen*, et al.* 2000). As with the thymidine analogues expression, Ki67 expression was predominantly located at the periphery of neurospheres.

These findings suggest that the neurosphere is a dynamic environment, with continuous proliferation at the peripheries and cell movement within it. The presence of both the immature glial marker GFAP and early neuronal marker Tuj in these peripheral zones of the neurosphere is consistent with the hypothesis that ENS progenitors are/arise from GFAP+ve glia (Liu*, et al* 2009, Joseph*, et al* 2011, Laranjeira*, et al* 2011). Experiments looking at the temporal relationship of EdU incorporation and expression of immature and mature glial/neuronal markers would help to confirm this hypothesis, however, as this was not part of the original aims it was outside the remit of this chapter.

Whether the differentiation state of a cell determines its location within the neurosphere or whether the location of a cell influences its differentiation remains to be determined. Possible factors that may influence this process include cell-cell interactions, which may change depending on a cells position within the neurosphere, or external factors such as availability of nutrients or oxygen tension. Although studies in adipose tissue-derived neurospheres have found that low oxygen tensions promote the maintenance of progenitor populations (Chung*, et al.* 2014), given the peripheral location of proliferating cells in ENS neurospheres such a role for oxygen tension seems very unlikely.

With regard to the comparison between neurospheres derived from embryonic mouse and postnatal human tissue the most striking observation was the proportion of cells incorporating EdU within the human neurospheres was dramatically less than those derived from embryonic mouse colon. This was the first obvious difference in behaviour between neurospheres derived from each species, but it remained to be established whether these were due to the species difference, or the developmental maturity of the source tissue.

3.3.4. Rate of proliferation in neurospheres is determined by the developmental maturity of the cells

Given the findings in 3.3.5., experiments were conducted to determine whether the species (mouse vs. human) or age (embryonic vs. post-natal) effect impacted on the rate of proliferation in neurospheres. The experiments found that only the developmental maturity of the source tissue from which neurospheres were derived and not the species was found to have a significant effect on the rate of proliferation. This finding is not surprising as many studies of the developing CNS have shown that proliferation of neural progenitors predominantly occurs in the embryonic period, and furthermore, the small amount of proliferation that does occur post-natally also declines with age (Kuhn*, et al.* 2011, McGinn*, et al.* 2012, Tan*, et al.* 2013). However this finding, coupled with the observation that there was no significant difference between neurospheres derived from postnatal day 28 mice and human samples taken from 3 month old infants is of importance. These results suggest that although neurospheres derived from embryonic mouse are not significantly different in their expression of typical ENS markers to their postnatal human counterparts there is a fundamental difference in their behaviour with regard to proliferation. It is therefore difficult to use results based on neurospheres derived from embryonic cells to predict behaviour of postnatal cells because of this fundamental

difference in proliferative behaviour. As most clinical potential clinical therapies are focused on using progenitors isolated from postnatal human sources this brings into question the use of embryonic mouse neurospheres in studies designed to further our understanding of the behaviour and indeed future safety of these cells.

3.4. Conclusion

The work within this chapter has found that in ENS neurospheres proliferating cells are predominantly to be found at the periphery, and this finding is the same in neurospheres derived from both mouse and human sources. Similarly, no significant differences could be identified in expression of typical ENS markers (P75, GFAP, S100) in neurospheres derived from either source.

Significant differences were identified in the behaviour of these cells depending on the developmental maturity of the donor tissue, with levels of proliferation seen within embryonic neurospheres up to five times greater than those seen in post-natal neurospheres. This raises questions as to the validity of using embryonic mouse neurospheres as a model for future human transplantation.

Both proliferation within the microenvironment of the neurosphere and the rate of neurosphere formation appear to be dependent on the relationship with surround cells. Therefore, given the known involvement of the Notch signalling pathway in the regulation of proliferation and its strong dependence on cell-cell interactions further study should be directed into the role of Notch in the control this behaviour.

Chapter 4: The Role of Notch Signalling in Human ENS Derived Cells

4.1. Introduction

4.1.1. Background

Studies have highlighted the potential for autologous transplantation of ENSPC to improve the function of aganglionic bowel in HSCR (Lindley*, et al* 2008). One of the crucial questions that needs to be answered before we can progress towards clinical trials is what are the mechanisms that regulate progenitor cell proliferation, self-renewal and differentiation within the neurosphere niche? During the routine isolation and culture of ENSPC we have noted the dramatic change in behaviour when ENSPC are removed from the microenvironment of the human bowel, moving from an essentially quiescent state to that of rapid proliferation. If this proliferation continues in an unregulated manner post-transplantation there remains the significant risk of tumour formation. Studies have shown that the Notch signalling pathway is involved in the regulation of neural progenitors outside of the ENS (Wakamatsu*, et al.* 2000, Taylor*, et al.* 2007, Tsarovina*, et al.* 2008). In addition there is evidence, limited to mouse models, suggesting that Notch signalling is necessary for normal ENS development (Okamura*, et al* 2008, Ngan*, et al* 2011) (See 1.1.4). However, the role of Notch signalling in mature human ENS neural progenitors remains to be established (see also 1.4).

4.1.2. Attribution of work in this chapter

Miss S Darling, a postgraduate MRes student, whilst working under my supervision assisted with the siRNA experiments detailed within this chapter. All other work, including conception, planning and interpretation of results were performed by myself. Part of the work in this chapter has been published in Plos One (Theocharatos*, et al* 2013)

4.1.3. Aims

Given the importance of understanding the mechanisms that regulate the behaviour of neural progenitors within the ENS the aim of this chapter is to determine whether the Notch signalling pathway regulates the proliferation and neuronal differentiation of mature human ENSPC in-vitro, and to begin to elucidate the state of Notch signalling *in-vivo*.

4.2. Results

4.2.1. Key components of the Notch signalling pathway are present in both human post-natal bowel and neurospheres.

Frozen ganglionic colonic sections were fixed and processed immediately after isolation. Using immunofluorescence and confocal microscopy, all four of the known mammalian Notch receptors were found to co-localise with components of the ENS (Figure 4.1) with the most prominent expression seen for Notch 1 and Notch 4. Expression was less widely seen for Notch 2 and 3. The antibodies used for the Notch receptors recognise both the inactive and activated components of the receptor, therefore no comment can be made on the activity of Notch signalling only the presence of its components in-vivo.

Similarly, when primary neurospheres were generated from colonic biopsies from the same patients, expression of all four Notch receptors was identified (Figure 4.2). When neurospheres were examined through their central section the localisation of expression was predominantly found to be at the periphery of the neurosphere, and with the exception of Notch 3, which had expression throughout, neurospheres demonstrated a marked decrease in expression at their centres. Confocal images of cells dissociated from such neurospheres also expressed the Notch receptors in 80-90% of cells. The appearance of the immunoreactivity at the cellular level was predominantly punctate, seen both in the cell membrane and within the intracellular domain. It should be noted that the antibodies used detect Notch do not distinguish between activated and inactive states of the receptors.

Having confirmed the presence of Notch receptors both in-vivo and in-vitro, we then moved on to analyse the expression of the common Notch ligand Jagged 1, which has previously been shown to be present in the murine ENS (Okamura*, et al* 2008) (Figure 4.3).

Jagged immunoreactivity was expressed in a pattern similar to that seen with the Notch receptors co-stained with the mature neuronal marker Hu in human colonic biopsies.

Figure 4.1. Expression of Notch receptor markers 1-4 in human colonic tissue.

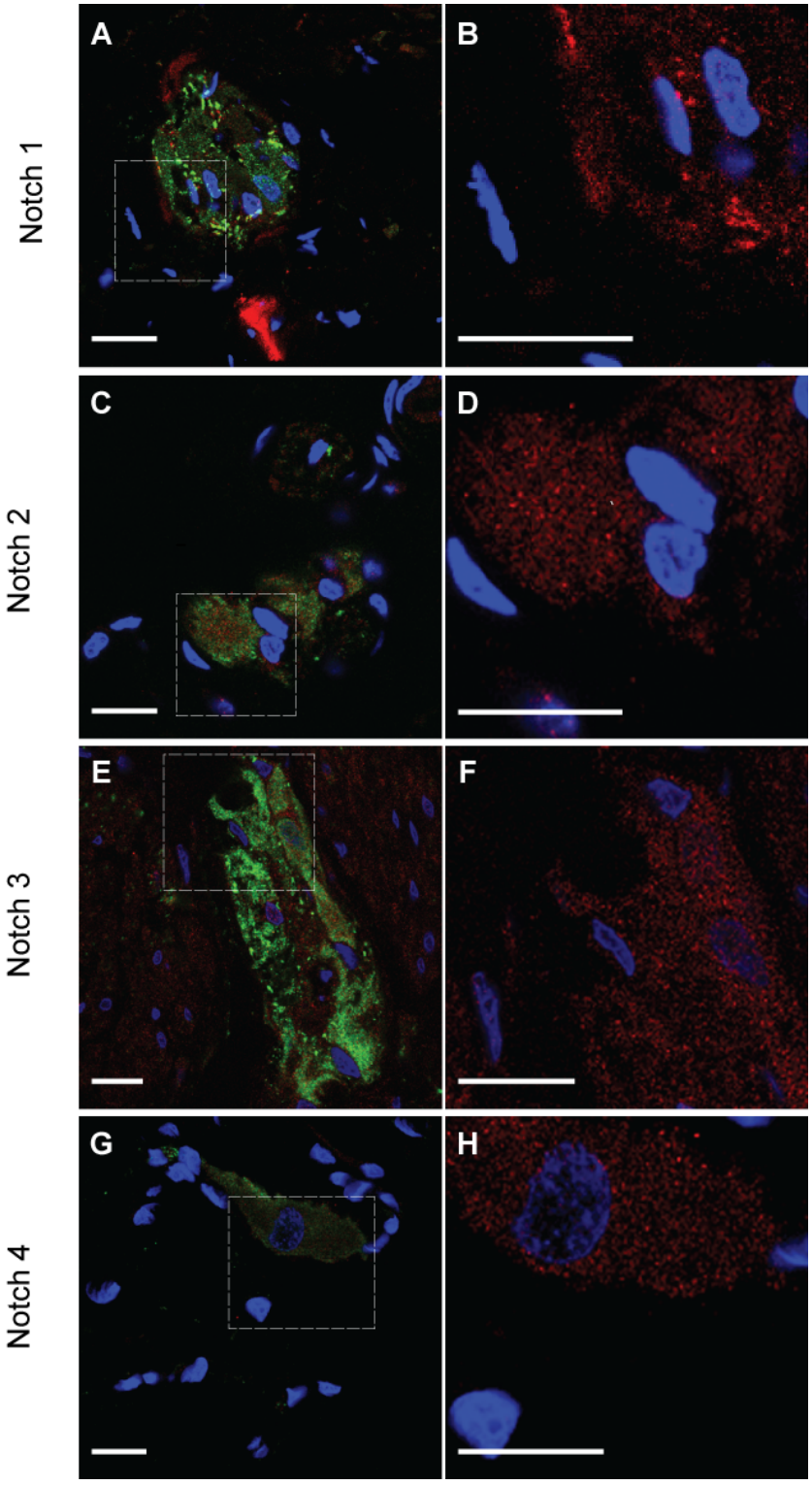


Figure 4.1. Confocal immunofluorescence of fixed frozen sections of the colonic myenteric plexus from control patients (3 month old infants with previous anorectal malformations). Antibodies used were against the neuronal marker Hu (green), Notch receptors 1-4 (red) and DAPI (blue). **B,D,F,H** show higher magnifications of the insert boxes drawn on A,C,E,G respectively with the Hu signal removed to clearly display expression of Notch receptors. Scale bar = 25µm

Figure 4.2. Notch receptors 1-4 are expressed in human neurospheres

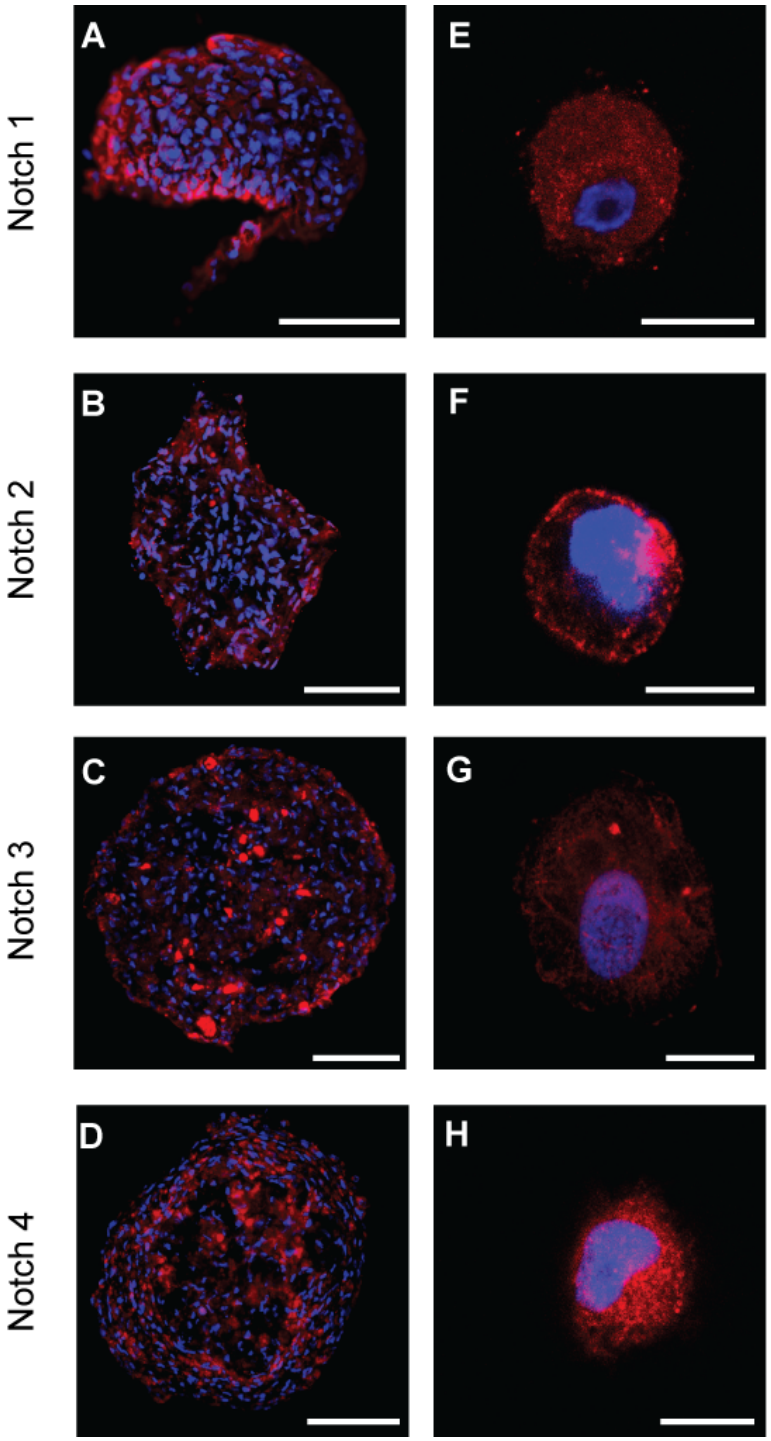


Figure 4.2. 7µm frozen sections of 28d old human neurospheres derived from the same patients as Fig 4.1. **A-D:** Standard immunofluorescence images for antibodies against the Notch receptors (Red) and Dapi (Blue). Scale bar A-D = 100µm **E-H:** Confocal images of single neurosphere cells isolated from the same patients fixed 3h after dissociation and stained as per A-D. Scale bar = 10µm

Of note, as previously described, high levels of expression were found on the endothelium of blood vessels within the colonic mucosa and muscle wall, which was also seen with Notch 1 (Sander*, et al* 2004). Within neurospheres, the pattern of Jagged 1 expression was similar to that of Notch 1,2 and 4 with a more pronounced expression in the periphery than that seen with the Notch receptors. Almost all cells demonstrated expressed of Jagged 1, with the cells within the first few layers at the periphery of neurospheres showing much higher levels of expression.

Figure 4.3. Expression of Jagged 1 in-vivo and in neurospheres.

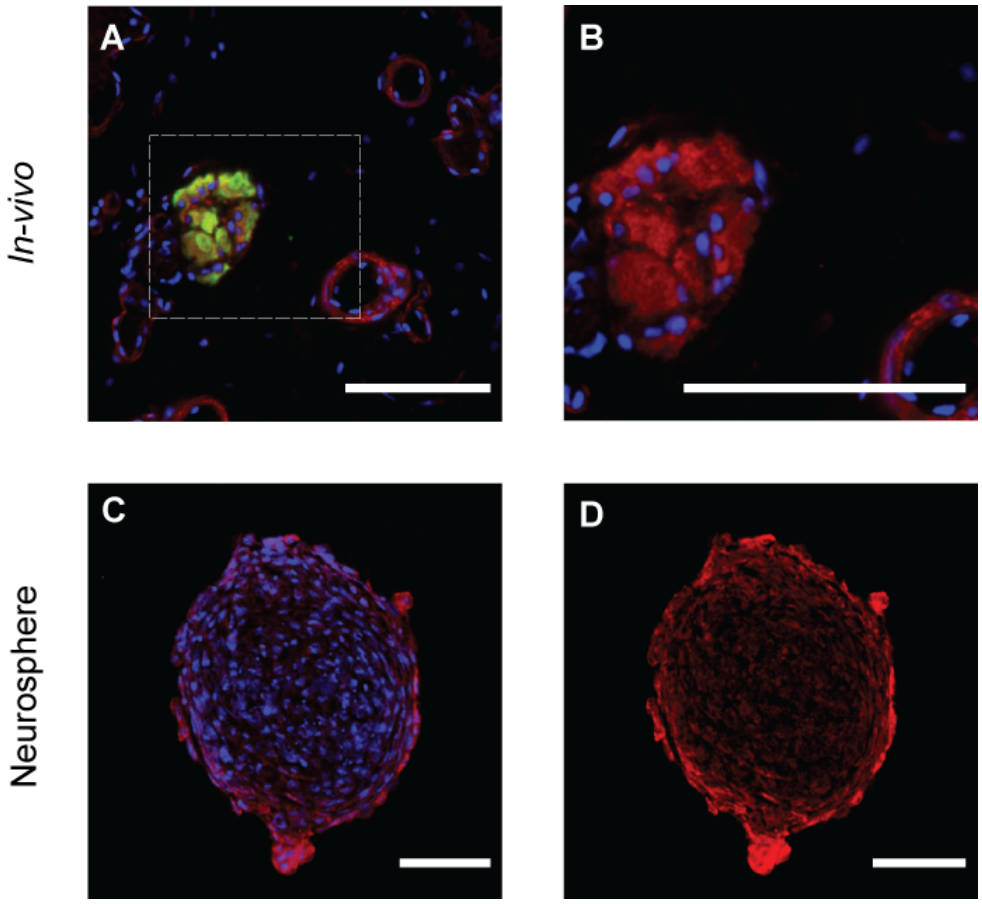


Figure 4.3. Frozen sections were prepared as previously. **A:** Immunofluorescence image from a colonic sections with antibodies against neuronal marker Hu (green), Jagged 1 (Red) and Dapi (Blue). **B:** Higher magnification image of insert shown in **A**. **C-D:** Immunofluorescence images from neurospheres generated from the same patients with antibodies against Jagged 1(Red) and Dapi (Blue). Scale bar = 100µm.

4.2.2. Inhibition of the Notch signalling pathway inhibits cell proliferation and promotes neuronal differentiation

Preliminary experiments to investigate the effect of Notch inhibition on human (HSCR) neurospheres were carried out using the γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT). Following a 4-day incubation period proliferation, as demonstrated by incorporation of the thymidine analogue EdU into nuclei, was found to be significantly reduced by more than 70% in the neurospheres exposed to DAPT compared with the DMSO vehicle controls (Figure 4.4 A,C,E).

Figure 4.4. Effect of DAPT administration on intact neurospheres.

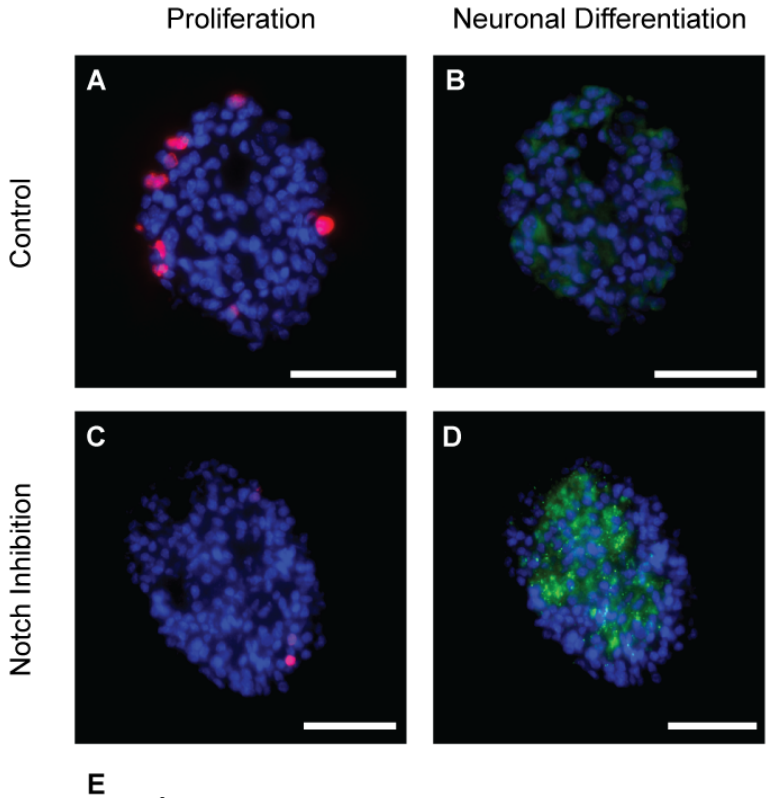


Figure 4.4. Human HSCR neurospheres were maintained under standard conditions for 10 days following their third passage before adding the Notch inhibitor DAPT (20uM) or DMSO to controls for a further 4d incubation. **A-D**: Frozen sections prepared as per previous figures. **A,C,E** demonstrates a significant decrease in the proportion of cells incorporating EdU (red) following DAPT administration (p=0.002). **B, D** shows that DAPT administration increases the expression of the early neuronal marker Tuj (green). Nuclei stained with dapi (blue). Scale bars = 50µm. Error bars represent standard error of the mean (SEM) from 3 experiments.

Conversely, the expression of the immature neuronal marker Tuj was seen to increase dramatically in its intensity and was predominantly found in the centre of neurospheres rather than the periphery (Figure 4.4 B,D). However, even with confocal microscopy it was not possible to give a quantitative result by analysing neurosphere sections as expression could not be localised to individual cells, as could be done with EdU which stains individual cell nuclei.

4.2.3. DAPT administration does not appear to reduce the proportion of proliferating cells by induction of apoptosis

Caspase-3 is part of the cysteine-aspartic acid protease family and is involved in both the extrinsic and intrinsic apoptosis pathways (Salvesen 2002) and its expression can be used as a marker for active apoptosis within a cell population. Therefore, expression of caspase-3 was analysed within neurospheres to investigate whether the reduction in the proportion of cells undergoing proliferation within neurospheres following Notch inhibition could at least in part be accounted for by an increase in apoptosis. Using the same conditions as 4.2.2, no significant increase in the expression of caspase-3 was identified in neurospheres cultured in the presence of the γ-secretase inhibitor in comparison to DMSO controls, either in terms of the percentage of the population expressing caspase 3(Figure 4.5) or the average cell number per high power field (hpf) (DMSO 8.9+/- 3.0 cells/hpf, DAPT 11.1 +/- 4.8 cells/hpf)

Figure 4.5. Effect of DAPT administration on apoptosis in human neurospheres.

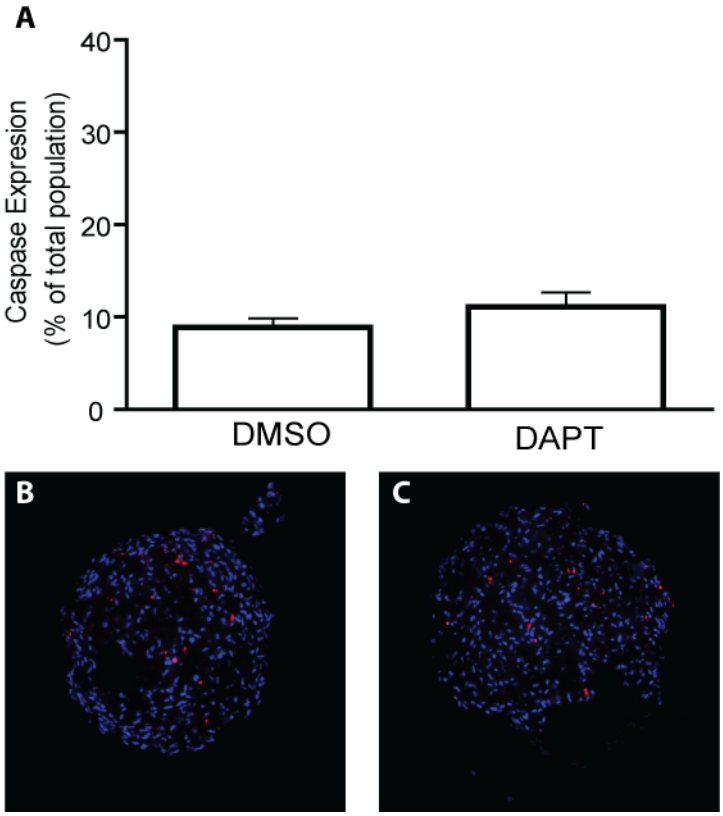


Figure 4.5. 7µm frozen sections were prepared from human neurospheres that had been incubated with DAPT or DMSO for 4d. **A:** No significant difference in the proportion of cells expressing caspase 3 was determined between either condition (p=0.4). Error bars represent SEM from 3 experiments.**B:** Representative section from a neurosphere under control conditions. **C:** Representative section from a neurosphere incubated with DAPT.

4.2.4. Dissociation of mature human neurospheres increases cell proliferation and neuronal differentiation.

To enable a quantitative assessment of the effect of Notch inhibition on neuronal differentiation human HSCR neurospheres were dissociated to allow expression of markers within individual cells to be determined. Therefore, the effect dissociating the cells on their behaviour, specifically proliferation and differentiation was first determined. The effect of dissociation was found to produce a linear relationship between the proportion of cells incorporating the thymidine analogue Edu and time following dissociation. After 8 days almost a third of the total population had were found to have proliferated (Figure 4.6 F). The increase seen in the proportion of cells expressing the early neuronal marker Tuj was similar following a linear relationship with time after dissociation, however the proportion of positive cells increased at more than twice the rate as seen with regard to EdU incorporation (Figure 4.6). Cells expressing Tuj tended to be located together (Figure 4.6 C and D). Ultimately, up to 50% of cells expressed the marker (Tuj) 8 days after dissociation.

Figure 4.6. Changes in proliferation and early neuronal differentiation over time in dissociated neurospheres.

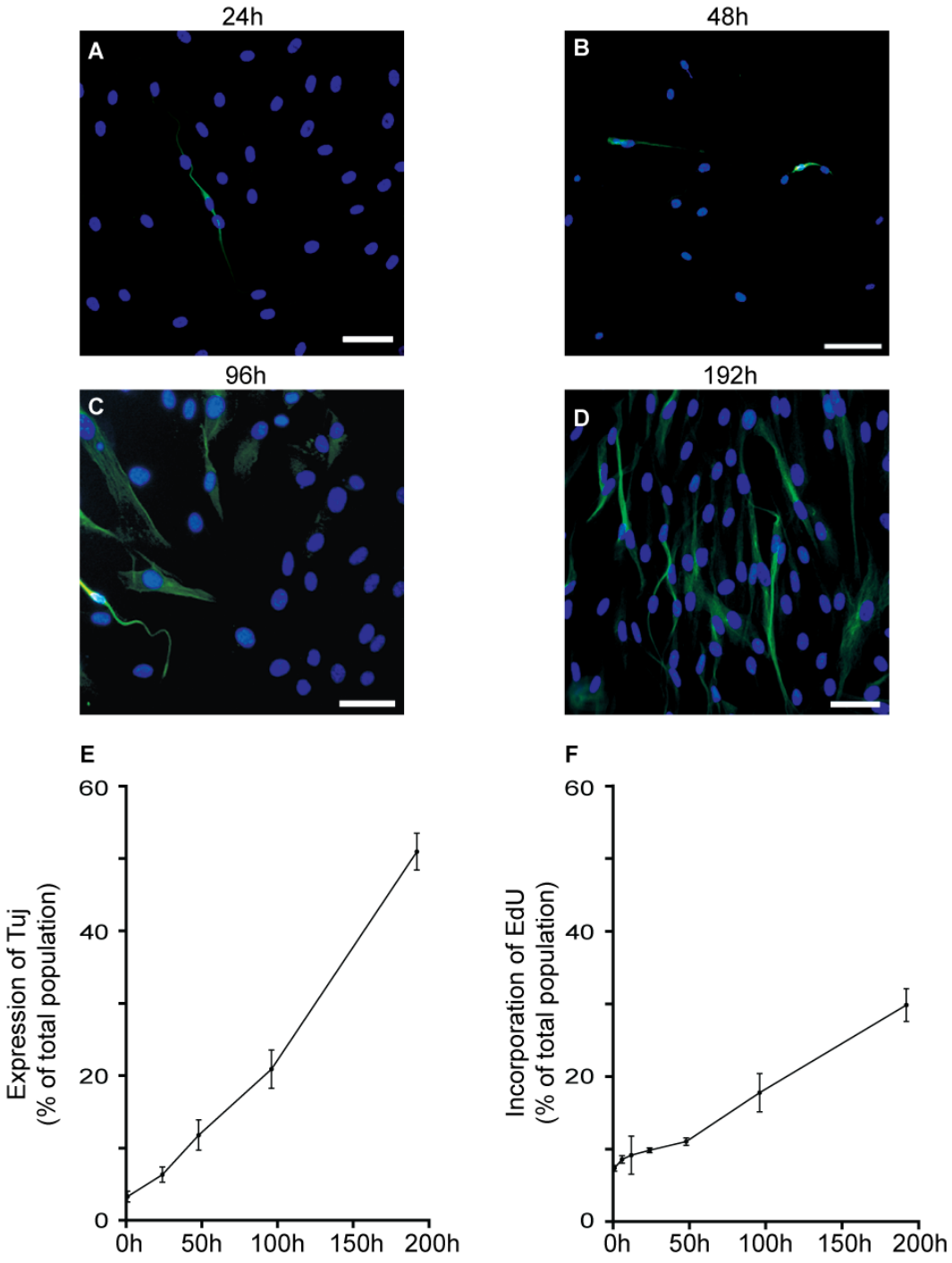


Figure 4.6. Mature human neurospheres were dissociated and cultured on chamber slides for up to 8 days. **A-E** demonstrate a steady increase in expression of the early neuronal marker Tuj (green) over time, with >50% of cells expressing Tuj by 192h (**D,E**, linear regression slope = 0.25, r^2= 0.72, p<0.001). Nuclei stained with dapi (blue). Similarly, **F** demonstrates a linear relationship between the proportion of cells incorporating EdU and time in culture, however the rate of increase is less than 50% of that seen with expression of Tuj (linear regression slope=0.12, r^2=0.9, p<0.001). Scale bars = 50µm. Error bars represent SEM from 3 experiments.

4.2.5. Selective siRNA knockdown of the canonical Notch pathway confirms the involvement Notch in regulating cell proliferation and neuronal differentiation.

Although the results of γ-secretase (DAPT) inhibition are consistent with a role for Notch signalling in the regulation of ENS progenitor cell proliferation and differentiation in neurospheres, they do not prove it because DAPT inhibition may affect other signalling pathways with which γ-secretase has been implicated (Groth*, et al* 2012). Therefore, in order to confirm that the decrease in proliferation and increase in neuronal differentiation previously seen (in 4.2.2) were due to Notch inhibition a selective siRNA knockdown was performed.

Predesigned, commercial siRNA oligomers (HsRBPJ-1/2/3 – see 2.4.2) were obtained against RBP-Jκ, a core component of the canonical Notch signalling pathway. To assess the efficacy of transfection a positive control (All Star®, Qiagen), which is known to induce cell death by targeting ubiquitously expressed genes which are essential to cell survival, was used. However, this was found to be unable to transfect central cells within human neurospheres. In contrast, in dissociated cells more than 80% of cells underwent cell death within 48hrs of transfection. Cell death was only seen in 5% of cells using a corresponding negative control. Therefore, to ensure an appropriate comparison could be made to those seen with DAPT administration in intact neurospheres, these experiments were repeated on dissociated neurospheres with similar results (Figure 4.7). Transfection of dissociated neurospheres with the HsRBPJ_3 oligomer was found to have an almost identical effect. Knocking down RBP-Jκ, resulting in an almost three-fold reduction in the number of cells incorporating EdU (Figure 4.8A-C). Furthermore, the knockdown promoted a dramatic, greater than five-fold, increase in neuronal differentiation to almost 50% of cells (Figure 4.8 A-D). Similar results were seen with two further siRNA oligomers HsRBPJ_1 and HsRBPJ_2 (data not shown).

The effectiveness of the knockdown was confirmed by a reduction of mRNA levels of RBP-Jκ with qRT-PCR using the ΔΔCt method, referencing to the housekeeping gene ß-Actin (see 2.8). Following siRNA treatment using the HsRBPJ_3 oligomer under the same conditions as above, mRNA levels of RBP-Jκ fell to one third of the level seen in human control neurospheres (Figure 4.8E).

Figure 4.7. Effect of Notch inhibition on cell proliferation in dissociated neurospheres

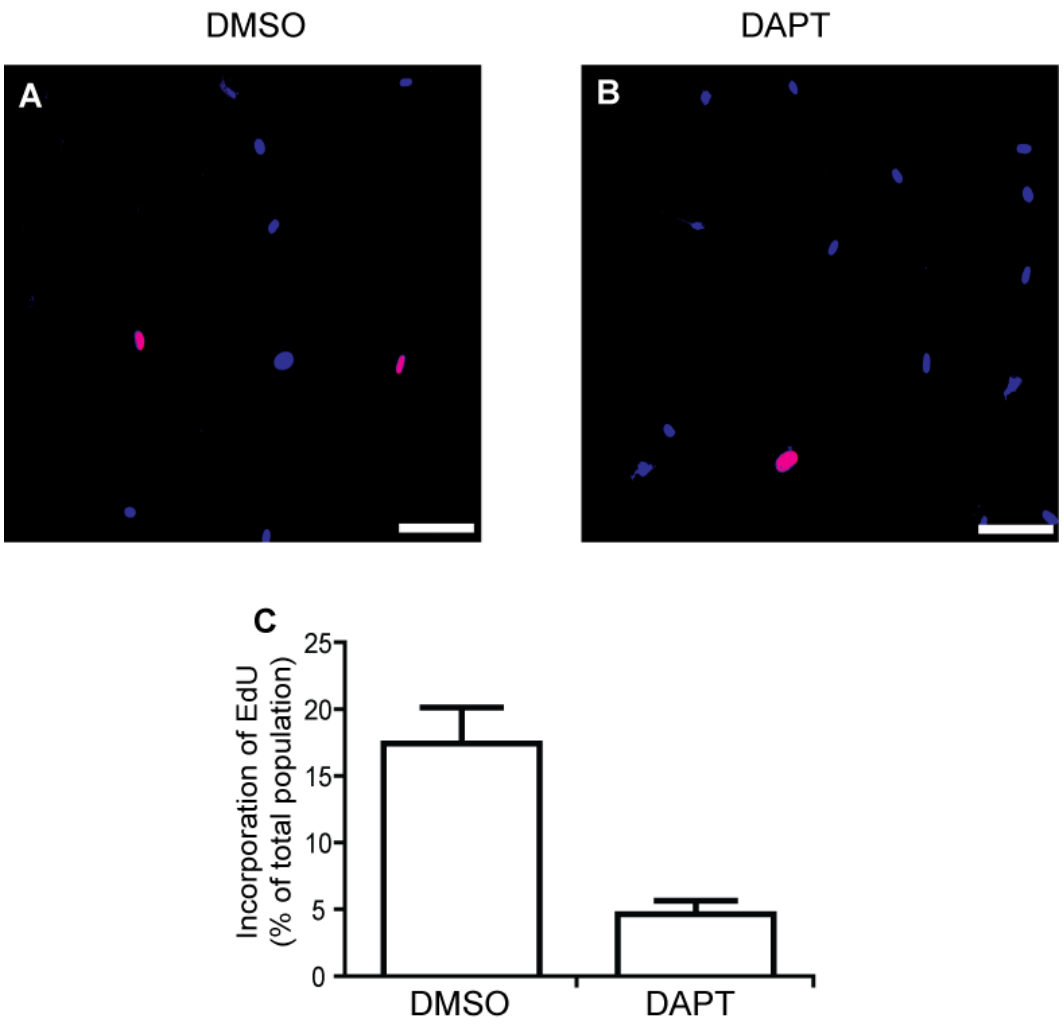


Figure 4.7. Human neurospheres were dissociated with trypsin and immediately plated onto fibronectin coated glass chamber slides in the presence of DAPT or DMSO control. **A-C** demonstrates a significant reduction in incorporation of EdU (red) in cells incubated with the Notch inhibitor DAPT ($p<0.0001$). Nuclei stained with dapi (blue). Scale bars = 50µm. Error bars represent SEM from 3 experiments.

Figure 4.8. Selective siRNA knockdown of Notch inhibits cell proliferation and promotes neuronal differentiation

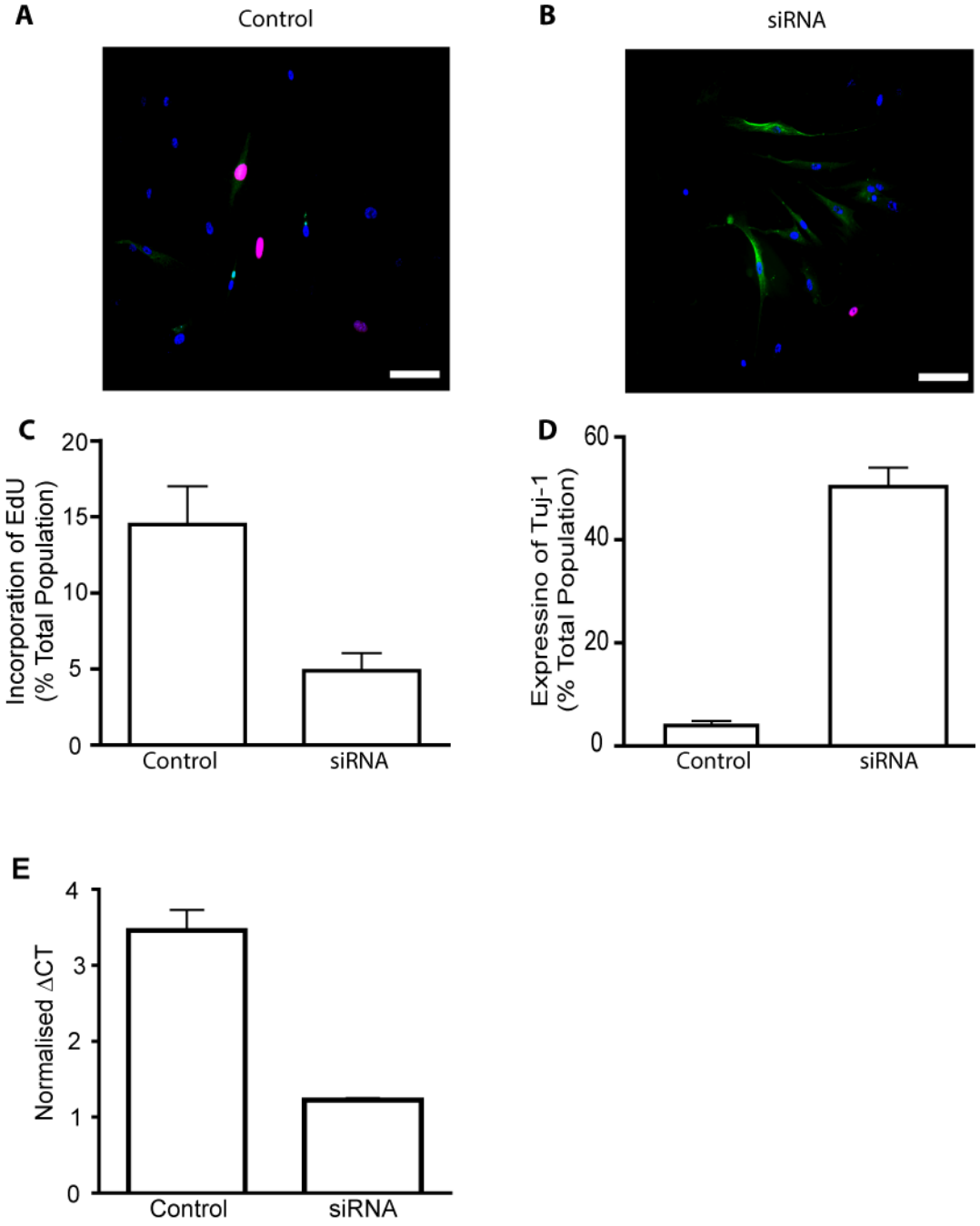


Figure 4.8. Dissociated human neurospheres were plated onto glass chamber slides (as per figure 3.5). 72h after transfection with either an siRNA against RBP-Jκ or a negative control cells were assessed for incorporation of EdU and expression the neuronal marker Tuj. **A-B:** Expression of Tuj (green) and incorporation of EdU (red) at 72h. Nuclei stained with dapi (blue). **C-D:** Quantitative confirmation that knockdown of RBP-Jκ results in a significant decrease in the incorporation of EdU and increase in expression of Tuj ($p<0.0001$). **E:** Confirmation of the specificity of knockdown. Three-fold reduction in the mRNA levels of RBP-Jκ following transfection with the HsRBPJ_3 oligomer ($p<0.001$). Scale bars = 50µm. Error bars represent SEM from 3 experiments.

4.2.6 Rate of neuronal differentiation is increased following Notch inhibition in cells dissociated from neurospheres.

Having determined the effect of dissociation on the proportion of cells from human neurospheres undergoing neuronal differentiation, the next step was to perform a more detailed analysis to identify the effect of Notch inhibition. Using DAPT inhibition over an 8-day time course experiment, Notch inhibition was found to significantly increase the proportion of cells from dissociated neurospheres expressing the early neuronal marker Tuj (Figure 4.9). However, this increase was less pronounced relative to the proportion of cells expressing Tuj under control conditions at the later time points (Figure 4.9 I).

Notch inhibition was also found to increase the proportion of dissociated cells expressing the more mature neuronal marker neuronal nitric oxide synthase (nNOS). However, there appears to be a lag time before the increase becomes significant (Figures 4.10). No significant increase in the proportion of cells expressing nNOS was seen at either the 24h or 48h time points. However, an increase was identified at both 96h and 192h, with almost one third of cells (32%) expressing nNOS at 192h. This represents a 40% increase in the proportion of cells expressing the marker relative to controls (Figure 4.10 E).

In contrast to the expression of nNOS, no significant increase in the expression of another mature neuronal marker ChAT is seen as a result of Notch inhibition at any time point (Figure 4.11). However, in both inhibited and control cells the proportion of ChAT did increase over time, with a 4-fold increase in expression seen during the first and subsequent 96h incubation periods.

Figure 4.9. Notch inhibition increases the rate of neuronal differentiation in dissociated neural progenitor cells

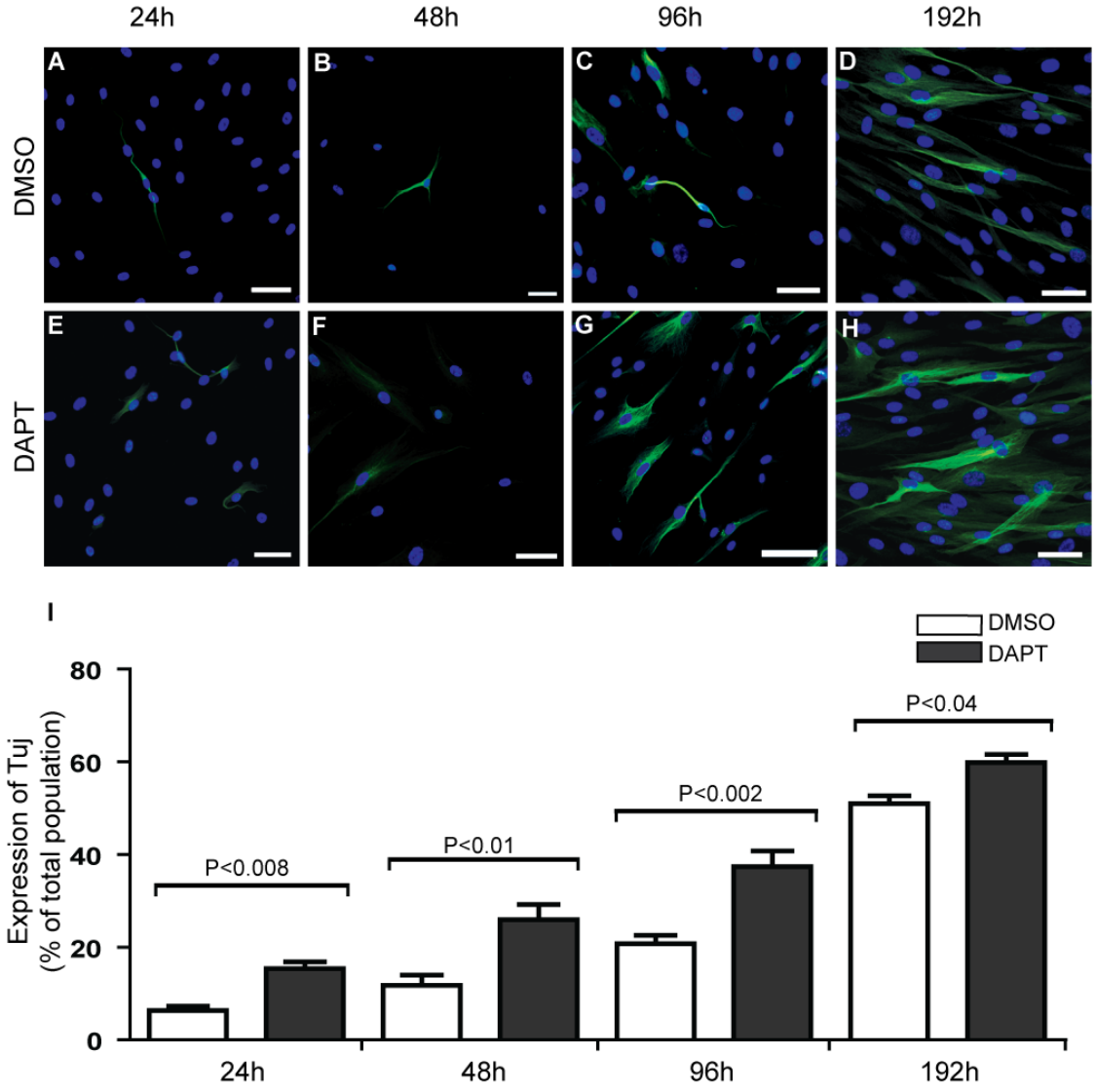


Figure 4.9. Mature human neurospheres were dissociated and cultured on chamber slides for up to 8 days either in the presence of the Notch inhibitor DAPT or DMSO control. **A-H** demonstrates a steady increase in expression of the early neuronal marker Tuj (green) over time in both conditions, but with significantly more in the Notch inhibited group. Nuclei stained with dapi (blue). **I:** The proportion of cells expressing Tuj is significantly higher at all time points in the Notch inhibited group. Scale bars = 50µm. Error bars represent SEM from 5 experiments.

Figure 4.10. Notch inhibition increases the expression of the mature neuronal marker nNOS

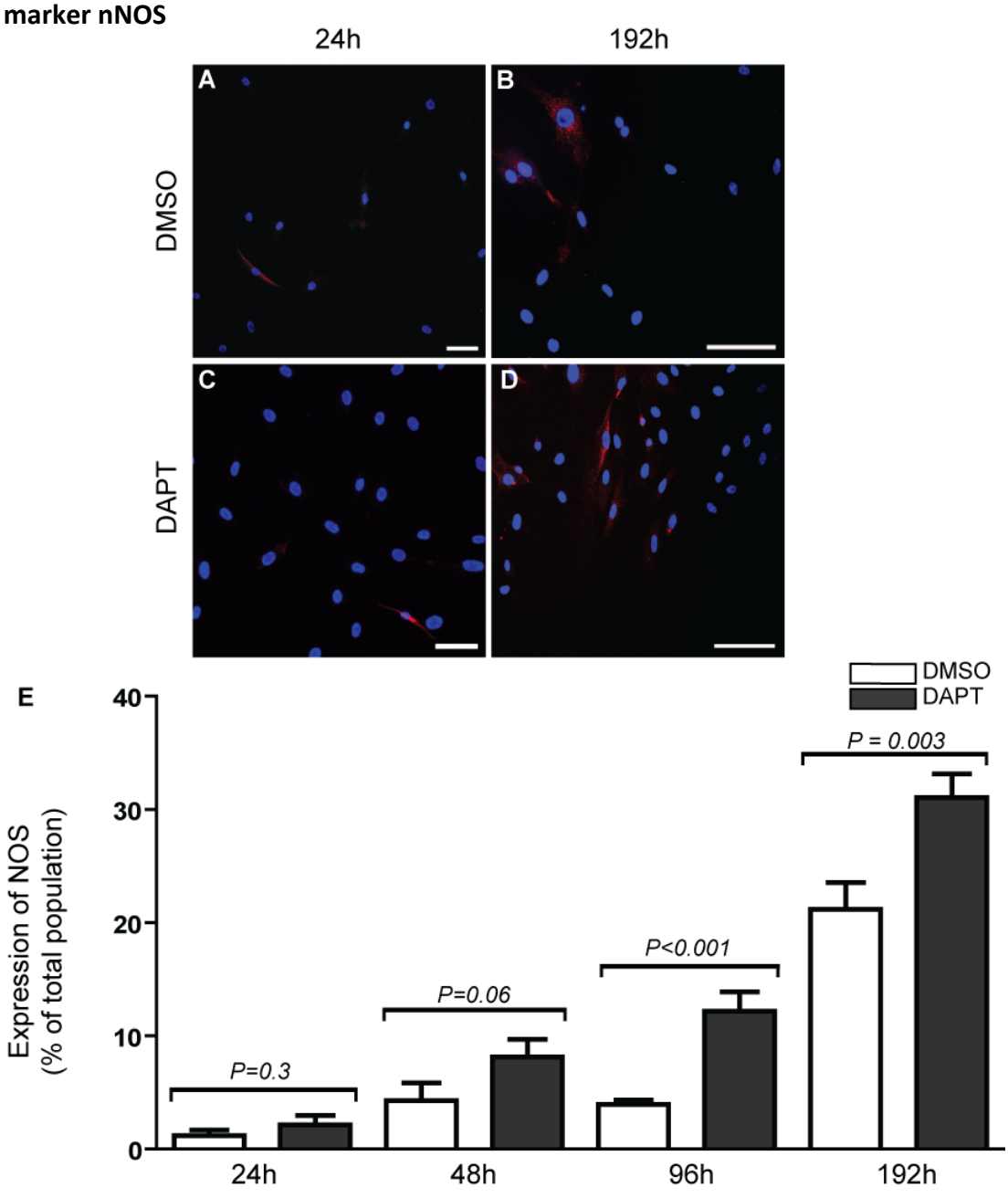


Figure 4.10. Mature human neurospheres were dissociated and cultured on chamber slides for up to 8 days either in the presence of the Notch inhibitor DAPT or DMSO control. **A-D** Demonstrate an increase in the expression of the mature neuronal marker neuronal nitric oxide synthase (nNOS, red) with a greater proportion of positive cells in the Notch inhibited group. Nuclei stained with dapi (blue). **E:** The proportion of cells expressing nNOS is not significantly higher in the Notch inhibited group until the 96h time point. A third of Notch inhibited cells express nNOS at 192h compared to less than a quarter of cells in the control population. Scale bars = 50µm. Error bars represent SEM from 3 experiments.

Figure 4.11. Expression of the mature neuronal marker ChAT increases with time in culture independent of Notch inhibition

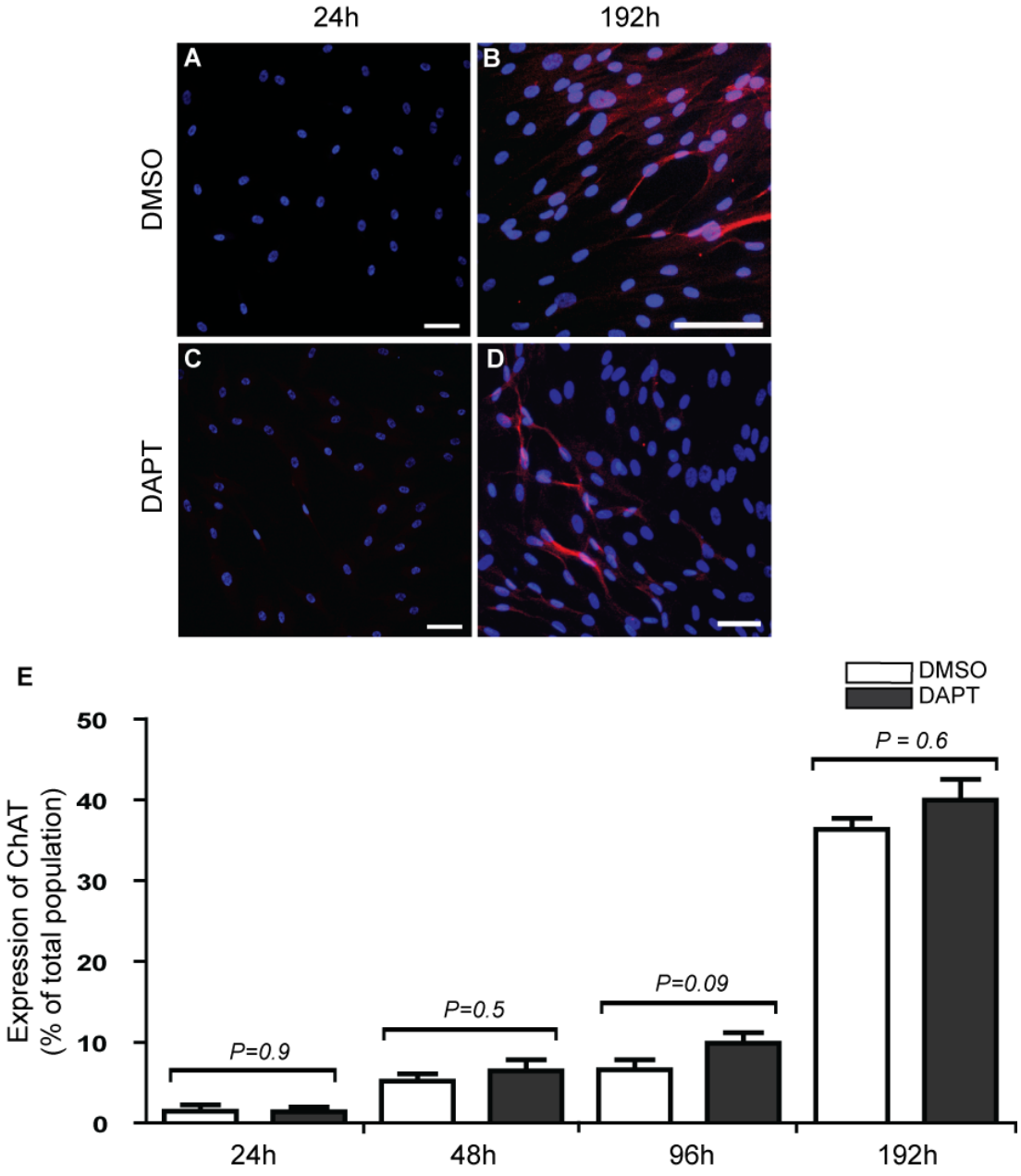


Figure 4.11. Mature human neurospheres were dissociated and cultured on chamber slides for up to 8 days either in the presence of the Notch inhibitor DAPT or DMSO control. **A-D** Demonstrate an increase in the expression of the mature neuronal marker choline acetyltransferase (ChAT, red) with time in culture. Nuclei stained with dapi (blue). **E:** The proportion of cells expressing ChAT is not significantly higher in the Notch inhibited group at any time point. Scale bars = 50µm. Error bars represent SEM from 3 experiments.

4.2.7. Notch signalling is disrupted by dissociation of neurospheres

Having demonstrated an increase in neuronal differentiation following either neurosphere dissociation or inhibition of the Notch signalling pathway we wanted to determine the activity of the Notch signalling pathway under both of these conditions. To do this we looked at the expression of the unbound Notch intracellular domain (NICD) within individual cells. In canonical Notch signalling activation of the pathway is initiated by corresponding ligand, which initiates the intracellular cleavage of the NICD, which in turn translocates to the cell nucleus to bind with RBP-Jκ and activate the downstream effectors (see 1.1.4). Therefore, using immunofluorescence techniques to identify the presence of cleaved NICD allows visualisation of cells in which the Notch pathway is activated.

Using an antibody against cleaved NICD at val1744,(Chen*, et al.* 2013), NICD expression was identified in more than 80% of cells in freshly dissociated neurospheres (Figure 4.12 A,D). Under control conditions the proportion of cells expressing NICD fell quickly to 35% by 12h, 20% by 24h and under 10% at 48h, after which point remained between 8-12% until 200h (Figure 4.12 D). Notch inhibition with DAPT accelerated this decrease, and low levels similar to those of the DMSO controls were maintained up to 200h (Figure 4.12 D).

Figure 4.12. Dissociation of neurospheres decreases the expression of NICD

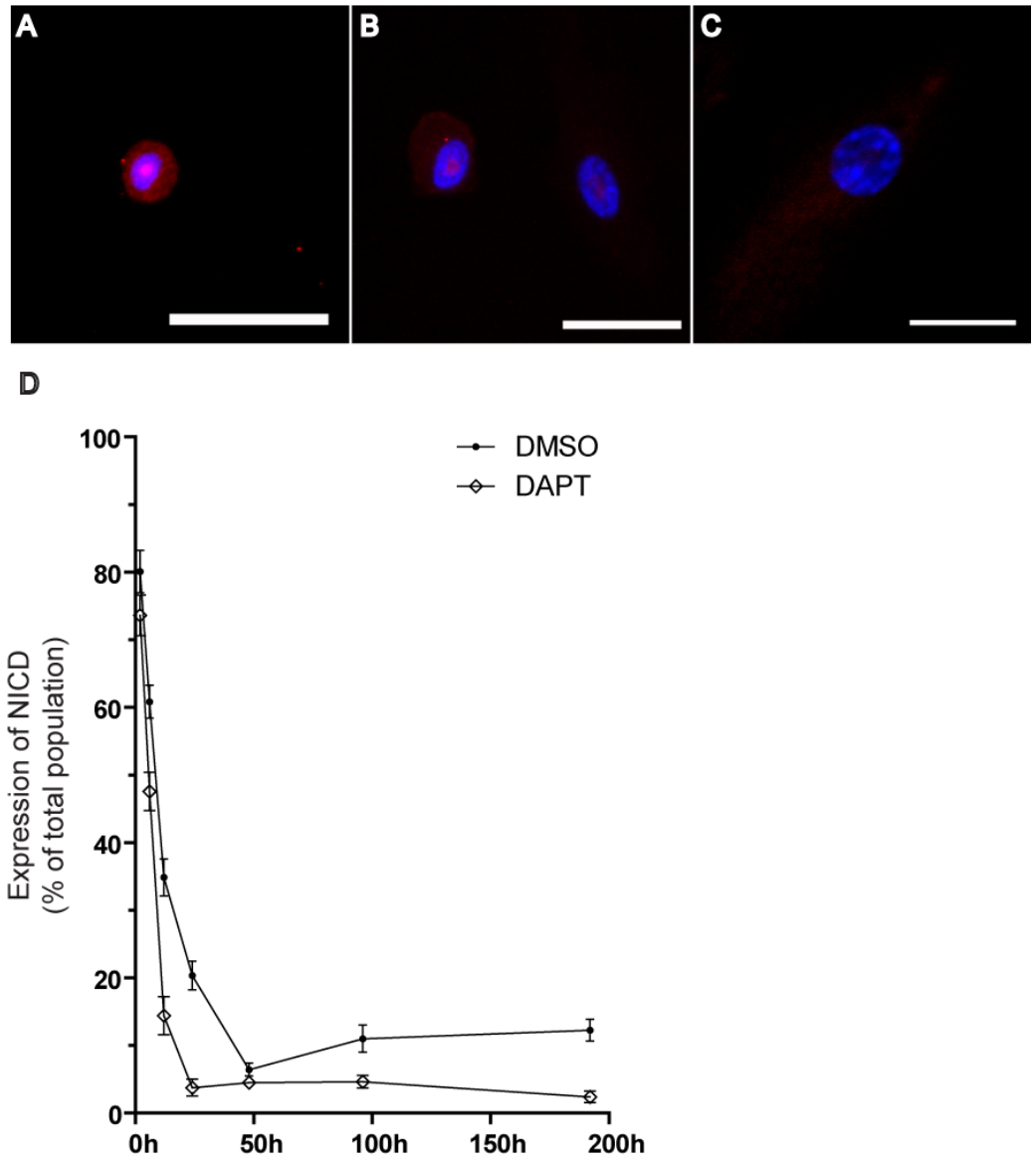


Figure 4.12. Neurospheres were dissociated, and the cells plated onto fibronectin-coated chamber slides and fixed at 1h, 6h, 12h, 24h, 48h, 96h and 192h time points. **A-B:** Demonstrate the loss of expression of cleaved NICD (red) following dissociation, 1h (**A**), 12h (**B**) and 24h (**C**). Nuclei stained with dapi (blue). **D:** Proportion of cells expressing NICD against time with and without Notch inhibition (DAPT). Scale bars = 50µm. Error bars represent SEM from 3 experiments.

4.2.8. Activated NICD is expressed in the ENS in-vivo and in-vitro

Given the changes in Notch activation as demonstrated by levels of NICD the next question was whether we could see any change in its activation state when isolating these cells from their original niche within the bowel and culturing them as neurospheres.

Looking at frozen colonic sections, fixed immediately after isolation, expression of NICD could be identified within elements of the ENS in-vivo (figure 4.13 A-D arrows). Similarly, the cleaved form of NICD was expressed in neurospheres cultured from biopsies taken from the same colonic sections. Consistent with localisation of both Notch receptors and ligands, the expression was predominantly found at the periphery of the neurospheres (Figure 4.13 E-F).

Figure 4.13. Expression of cleaved NICD in-vivo and in-vitro

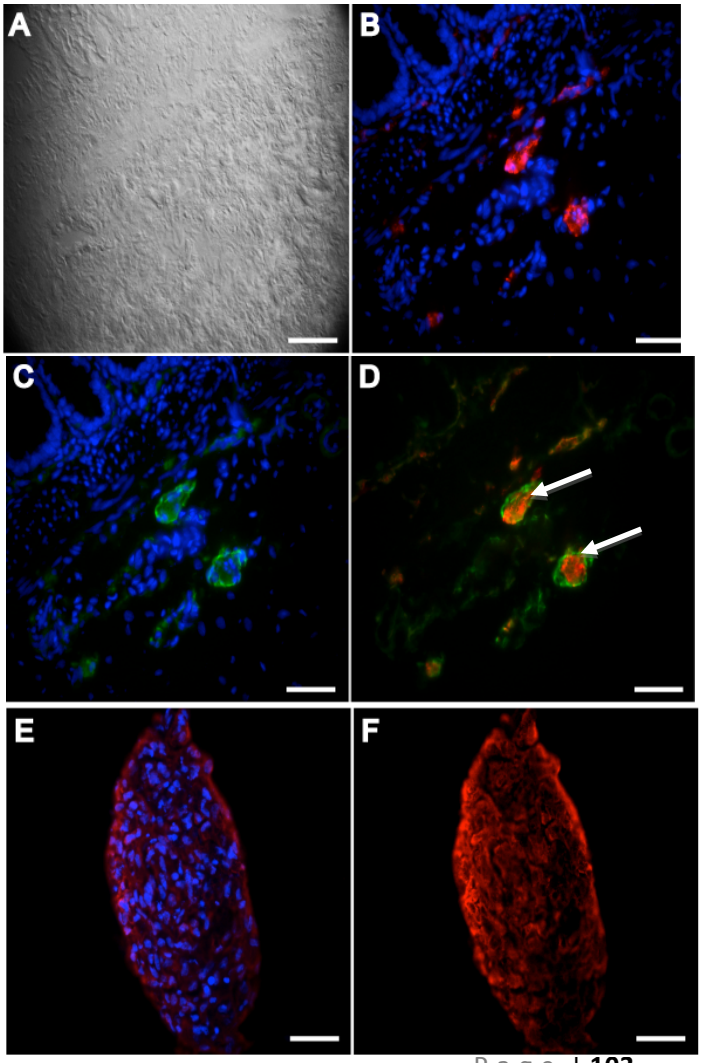


Figure 4.13. Expression of the NICD in human ganglionic colonic submucosa. A-D: Images from the same 7 µm colonic frozen section demonstrating NICD expression within components of the ENS. **A** Phase contrast. **B**: NICD expression (red), nuclei stained with dapi (blue). **C:** mature neuronal maker Hu (green). **D:** Merge.

E-F: 7µm central frozen section of neurosphere demonstrating NICD expression predominantly localised at the periphery. Scale bars = 50µm

4.3. Discussion

Given the dramatic change in behaviour that is seen in ENS progenitors when removed from their niche in-vivo, where only low levels of proliferation and neurogenesis are detected (Pham*, et al.* 1991, Li*, et al.* 2011) and brought into culture there is understandably a concern regarding any future therapeutic use of such cells. Therefore, the need to identify the mechanisms that regulate this behaviour is important to enable us to predict and indeed manipulate their behaviour prior to human transplantation. This chapter demonstrates that components of the Notch signalling pathway (receptors and their ligand, Jagged-1) are expressed in both the human post-natal ENS and ENS derived cultures in-vitro. Furthermore, this chapter confirms that Notch signalling is involved in the regulation of proliferation and neuronal differentiation in-vitro.

4.3.1. Notch ligands and receptors are present the human post-natal ENS

All four known notch receptors, Delta-like ligands and Jagged 1 and Jagged 2 have been shown to be present in the mouse/rat gut (Sander*, et al.* 2003, Sander*, et al* 2004, Okamura*, et al* 2008, Ngan*, et al* 2011). Hence it is of no surprise that we identified components of the Notch signalling pathway within the ENS of the postnatal human colon. No previous study has reported the presence of the Notch 3 receptor, most having focussed on Notch 1 and 2. Caution must be applied to our preliminary finding indicating the presence of Notch 3 due to the specificity of the antibodies used. Little detail is given from the manufacturers regarding the precise target for their anti-Notch 3 antibody and although we used IgG controls to determine the specificity of immunofluorescence (not shown), there remains the possibility that there was some cross-reactivity with other Notch receptors. Nevertheless, the experiments reported demonstrate that multiple components of the

Notch ligand/receptor system were present both in-vivo and in cultured ENS progenitors, and it was outside the scope of the present chapter to take this finding further.

Notch receptors and their ligands within neurospheres were found to be predominantly localised in the outer/peripheral layers of cells within neurospheres. The localisation of Notch signalling and indeed the behaviour of cells within neurospheres is not well documented either within the CNS or ENS. However, this finding is consistent with our findings in chapter 3 which found that proliferation in neurospheres is predominantly located at the peripheries, with more mature cells migrating towards the centre.

4.3.2. Notch inhibition reduces proliferation in human ENS progenitor cells

Notch inhibition, with the γ-secretase inhibitor DAPT was found to cause a significant reduction in ENS cell proliferation in-vitro, using the incorporation of the thymidine analogue EdU into nuclei to demonstrate DNA synthesis. A four-day treatment with DAPT resulted in a 70% reduction in EdU incorporation in both intact neurospheres and cells dissociated from them, which is consistent with previous studies in both the CNS (Gao*, et al* 2009, Imayoshi*, et al* 2010) and embryonic murine ENS (Okamura*, et al* 2008, Ngan*, et al* 2011). However, both the results of this chapter, using DAPT and those in the murine ENS leave open the possibility of the reduction in proliferation being caused by off-target, non-specific effects of inhibition. The γ-secretase complex inhibited by DAPT is known to be involved in the membrane proteolysis of more than 80 other substrates (Groth*, et al* 2012). Although previous findings within our lab confirmed that DAPT inhibition resulted in a down-regulation of the downstream Notch transcription factors Hes1 and Hes5 (Theocharatos*, et al* 2013), this still did not prove that the changes in cell behaviour are directly due to the inhibition of Notch signalling. Similarly, the approach of Okamura *et al* used a conditional knockout of the widely expressed glycosyl-transferase POFUT-1

(Okamura*, et al* 2008), the effects of which may not be restricted to inhibition of the Notch signalling pathway within the ENS.

This chapter provides definitive evidence that Notch signalling is required to maintain a proliferative state. Transfection with the Notch specific anti-RBP-Jκ siRNA was found to reduce the proportion of cells incorporating EdU by almost 70% at 72h. This selective approach confirms that the reduction in proliferation seen was due to Notch inhibition rather than other off-target effects.

The second assumption that required clarification was whether the decrease in number of cells incorporating EdU was due to a reduction in the rate of proliferation or whether it was due to an increase in apoptosis. One study looking at the affect of DAPT administration on human tongue carcinoma cells found a dose-dependent increase in apoptosis, as revealed by the expression of caspase-3 (Grottkau*, et al.* 2009).

The results in this chapter did not show a significant difference either in the number or proportion of cells expressing Caspase-3 expression in human ENS neural progenitors treated with DAPT. This is consistent with the findings of Okamura *et al*, who could not identify apoptosis following their model of Notch inhibition in the developing ENS (Okamura*, et al* 2008). Although caspase-3 expressed is not identified in all apoptotic cells, it was decided not to pursue this exhaustively as there were no other indicators to suggest on going cell death within the neurospheres, i.e. a decrease in size of neurosphere over time in culture in the presence of DAPT.

4.3.3. Notch inhibition increases the neuronal differentiation human ENS neural progenitor cells.

Inhibiting the Notch signalling pathway with either the γ-secretase inhibitor DAPT or following siRNA knockdown of RBP-Jκ was found to result in an almost 8-fold increase in the expression of the immature neuronal marker Tuj, with positive cells demonstrating a typical neuronal phenotype. Unsurprisingly, this increase mirrors the findings in ENS mouse models in which Notch is inhibited via either a PTCH1 or POFUT1 knockout confirming that these studies were reflecting the consequences of Notch inhibition rather than some other unidentified effect of the knockouts (Okamura*, et al* 2008, Ngan*, et al* 2011).

Importantly, Notch inhibition also promoted the expression of the more mature neuronal marker nNOS. Unsurprisingly this lagged behind the expression of the immature marker Tuj, with nNOS showing a significant increase after 96h compared to 24h. The overall expression of ChAT was not significantly higher in Notch inhibited cells than in controls after the full 192h incubation period, even though there had been a significant increase in the proportion of cells expressing ChAT (4-fold increase between 96h and 192h).

The development of nitrergic and cholinergic neurons is known to differ. During embryonic development ENS cholinergic neurons differentiate and exit the cell cycle earlier than their nitrergic counterparts (Pham*, et al* 1991). Although, there is a paucity of evidence detailing the timing of nitrergic and cholinergic differentiation in-vitro, the literature suggests that nitrergic differentiation may occur earlier. Papers report increases in the expression of nNOS after a period of 24-48h (Anitha*, et al.* 2010), whereas increases in ChAT are reported after longer time periods, e.g. 18 days (Nilbratt*, et al.* 2010). However, these observations must be treated with caution as there are no studies providing a direct comparison between cholinergic and nitrergic differentiation in-vitro under controlled conditions.

It has been shown that neuronal subtypes respond differently to inflammatory insults within the gut (Winston*, et al.* 2013)and it is equally plausible that these neuronal subtypes respond differently to changes Notch signalling. Preliminary evidence from Sander *et al* provides some support for this premise, in finding differential expression of the Notch 1 receptor between enteric cholinergic and nitrergic neurons. However their report was limited to in-situ hybridisation results of one single Notch receptor and did not attempt to correlate these findings with function.

It is not clear from these results whether the Notch is just responsible for promoting the initial switch to an immature neuronal lineage, or whether prolonged inhibition is required to promote mature neuronal differentiation. There is not enough evidence provided in this chapter to draw any definitive conclusions as to whether these results are due to differences in the timing of differentiation or the response to changes in Notch signalling. However, it does warrant further study because if it is possible to promote the differentiation of inhibitory neurons in progenitors prior this may improve the functional response of any future cell-based transplantation therapies.

4.3.4. Notch signalling is disrupted by dissociation of neurospheres

In this chapter we have used standard immunofluorescence techniques to detect the cleaved form of NICD, to determine the activation state of the Notch signalling pathway. We found that the proportion of cells in which NICD could be detected fell quickly after dissociation, a process that was accelerated by Notch inhibition. This decrease in the expression of cleaved NICD following dissociation is not unexpected. Notch signalling is classically activated by transmembrane ligands on adjacent cells, hence disruption of cell-cell interactions will interrupt canonical Notch activation. Following dissociation, the proportion of cells expressing NICD under uninhibited conditions gradually increases again,

as the cells become more confluent. Although, the expression of NICD does fall following either cell dissociation or Notch inhibition it is not immediate. Other groups have used the expression of NICD as a proxy for Notch activation (Hansson*, et al.* 2006, Del Monte*, et al.* 2007), however it still has its drawbacks, the detection of cleaved NICD may lag behind the true activation state, for example, although the antibodies only detect the cleaved form of NICD, it is unclear how long NICD may remain detectable following cleavage before it is broken down and recycled. Furthermore, the Notch signalling pathway can be activated in a non-canonical, NICD independent, manner (Martinez Arias*, et al.* 2002), which this technique would not detect. In order to more precisely determine the changes in Notch activation 'live-reporting' techniques such as using a luciferase-base reporter to assess levels of activated RBP-Jκ would be required (Ilagan*, et al.* 2011).

The presence of cleaved NICD was also used to determine activation firstly within the microenvironment of the neurosphere, where its distribution is predominantly in cells at the periphery, suggesting that Notch signalling is most active in this region, which itself is consistent with the previous findings regarding the expression of Notch receptors and ligands (4.2.1). Secondly, it is also seen within the ENS in-vivo*,* which is more surprising. The initial hypothesis was that Notch was inactive in-vivo, and following an 'injury', such as isolating neural progenitors, it became active thus explaining the increase in proliferation and maintenance of a stem cell like state. If cleaved NICD is present in-vivo this may suggest that Notch is necessary but not sufficient for progenitor cell self-renewal in-vivo. More importantly, it suggests that the environment in-vivo exerts a dominating inhibitory action on ENS progenitor cell self-renewal, irrespective of the activity of the Notch signalling pathway.

4.4. Conclusions

The work within this chapter confirms that Notch signalling plays an active role in the maintenance of a proliferative, progenitor-like state within post-natal ENS. It is clear that although Notch signalling is required it is not sufficient to fully explain the regulation of ENS progenitor behaviour, which is consistent with our growing understanding of the complexity of both Notch signalling and cell regulation. It still remains to be established what other factors play a role, but the work here provides a basis for future experiments to determine firstly, whether Notch signalling regulates ENS progenitor behaviour post transplantation and secondly, whether this can be manipulated to improve both the effectiveness and safety of any future therapy.

Chapter 5: Development of a mature human colonic smooth muscle model

5.1. Introduction

5.1.1. Background

Previous work has demonstrated the therapeutic potential of using ENPSC as an autologous transplant therapy for HSCR. Studies have shown their ability to migrate and form new neurons post-transplantation in-vivo, and within lab models restore a normal pattern of contraction (Dong*, et al* 2008, Lindley*, et al* 2008, Hotta*, et al* 2013). The major limitation of these studies is that they have all used animal tissue as the host for transplanted ENSPC. Therefore, although providing encouragement that transplantation may provide a therapeutic benefit, questions still remain with regard to how these cells might behave in the microenvironment of the mature human colon. Although, ENSPC derived from patients with HSCR can be transplanted into laboratory-based animal models (Lindley*, et al* 2008), currently they cannot be injected into mice with a HSCR phenotype because of issues with rejection. Given observations such as the dramatic change in behaviour seen from an essentially quiescent state in-vivo to that of uncontrolled growth and differentiation in tissue culture, there is a need to predict, as closely as possible what will happen to the behaviour of ENSPC when transplanted back into the environment of the human bowel. Will these cells come back under control of the host environment or will they continue to proliferate and develop into potential tumour?

Currently it is not possible to maintain human bowel explants in culture for much longer than 24h, predominantly due to the limited diffusion of oxygen and nutrients beyond a thickness of 2mm (Griffith*, et al.* 2005). The development of an ex-vivo mature human

colonic smooth muscle model (HCSMM) that could be maintained in culture for prolonged periods of time would provide the ability to observe the behaviour and the functional effect of transplanted human ENSPC in the microenvironment of aganglionic human smooth muscle. This would provide a stepping-stone between the existing animal models and future human clinical trials enabling us to more accurately predict both the potential safety and functional outcome of autologous transplantation of ENSPC for HSCR.

5.1.2. Attribution of work contained within this chapter

Functional physiological assessment of HCSMMs for smooth muscle activity was carried out with Dr Rachel Floyd, University of Liverpool. All other work was carried out by the author.

5.1.3. Aims

Studies have demonstrated that it is possible to culture layers of physiological active human smooth muscle on a fibrin scaffold (Hecker 2005, Somara*, et al.* 2009). The aim of this chapter is to develop a HCSMM using aganglionic colonic smooth muscle in which the behaviour and functional response of transplanted ENSPC can be investigated. The model must: (1) Contain functional smooth muscle (2) Be reproducible (2) Be maintained in culture for >2 weeks (3) Allow physiological testing both pre and post ENSPC transplantation.

5.2. Results

5.2.1. Development of a fibrin based human colonic smooth muscle model

Following a review of the current literature it was decided to base the first attempts at developing a HCSMM on the model described by the group led by Khalil Bitar (Hecker 2005, Somara*, et al* 2009). 35mm standard cell culture dishes (Nalge-Nunc) were firstly coated with 1ml of Sylgard® 184 (Dow Corning) and a prepared 5mm diameter Sylgard cylinder was placed in the centre of each dish before allowing to cure for a minimum of 24h (Figure 5.1). Each dish was sterilised using a two-step 70% ethanol and UV light process, with a 1h incubation period for each step. Immediately prior to use, 500µl of FB medium, supplemented with both thrombin and fibrinogen (Sigma-Aldrich) at concentrations detailed in table 6.1, was added to each dish, briefly swirled and left for 15minutes at 37˚C for the fibrin to polymerise. A single cell suspension of aganglionic human colonic smooth muscle cells (2.3.1.3) was then seeded at concentrations again detailed in table 5.1.

The fibrin contracts to form a ring around the central pole on the dish over a period of 5-10 days, with the media being replaced every 48h (figure 5.1). Once a tight ring had formed the standard FB culture medium was changed to HS medium to promote mature smooth muscle differentiation. HCSMMs were maintained under these conditions until required for further testing or they became unstable (Table 5.1).

44 individual constructs were seeded, with the main finding following these Initial experiments that, despite attempted optimisation of the conditions, the overall reliability, i.e. the success rate of forming a stable ring by day 14 in culture was low 23/44 (52%, Table 5.1). Early attempts using either low thrombin (<10Units/ml), fibrinogen (2 mg/ml) or cell seeding densities below 150K cells/ml did not result in any intact constructs by day 14

(Table 5.1). Success rates were improved to 68% above these minimum thresholds, particularly when seeding at a density of 300K cells/ml (Table 5.1)

In an attempt to increase the number of mature smooth muscle cells maintained within the construct, and thereby increase the theoretical force that could be generated by the model the cell density was increased further to 400K cells/ml. However, this was associated with a further reduction in the reliability of forming a mature, stable ring in culture by 14 days.

Figure 5.1. Human colonic smooth muscle model using a fibrin scaffold

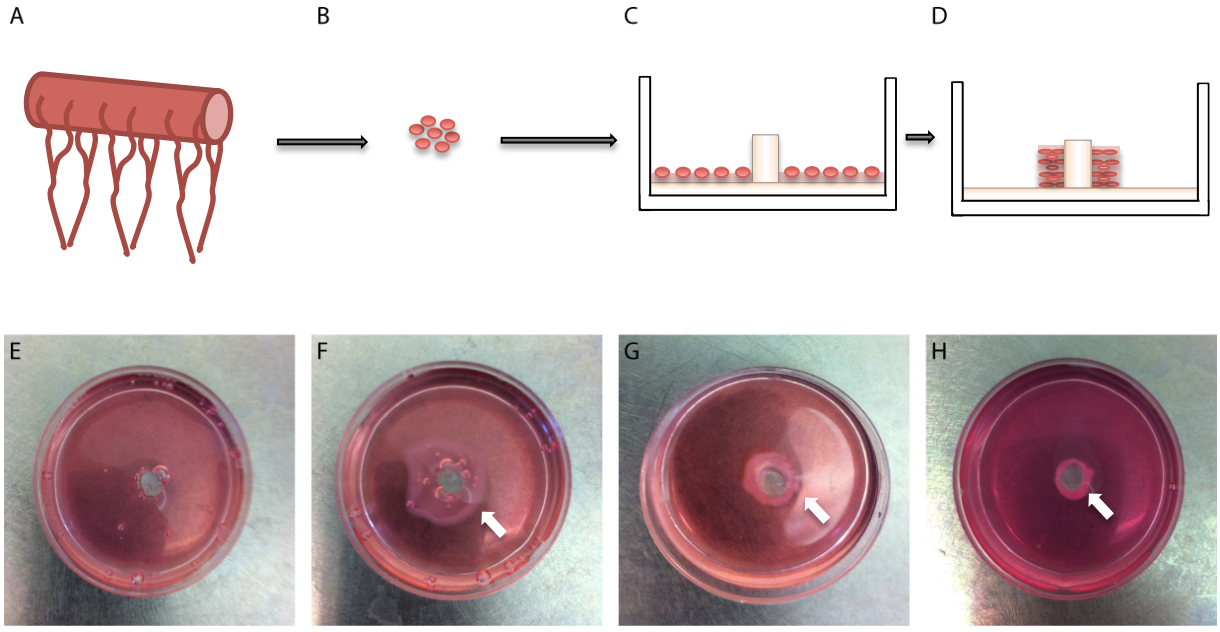


Figure 5.1. **A:** Human smooth muscle was isolated from colonic biopsies as previously described to generate a single cell suspension (**B**). **C**: Standard 35mm plastic dishes were prepared with a Sylgard® base and 5mm central pole, onto which a polymerised layer of fibrin was produced. The single cell suspension is then seeded onto the fibrin at varying concentrations (Table 6.1). **D-H:** As the fibrin scaffold contracts it forms a tighter ring embedded with smooth muscle cells around the central pole and this becomes the HCSMM. **E-H**: Represent a typical time course, **E** = 0h after seeding, **F** = 48h, **G** = 5d, **H** = 10d (white arrow highlighting the forming HCSMM).

Table 5.1. List of conditions and outcomes for each fibrin-based HCSMM

	Thrombin	Fibrinogen	Seeding Density	Patient	Ring formed	Outcome
1	10 Units/ml	2 mg/ml	200K/ml	H001	48h	Ring snapped at 16d
2	10 Units/ml	2 mg/ml	200K/ml	H001	-	Failed to form by 14d
3	10 Units/ml	2 mg/ml	200K/ml	H001	-	Failed to form by 14d
4	10 Units/ml	4 mg/ml	200K/ml	H001	48h	Electively sacrificed at 30d
5	10 Units/ml	4 mg/ml	200K/ml	H001	48h	Electively sacrificed at 70d
6	10 Units/ml	4 mg/ml	200K/ml	H001	-	Failed to form by 14d
7	10 Units/ml	4 mg/ml	200K/ml	H001	72h	Electively sacrificed at 30d
8	10 Units/ml	4 mg/ml	150K/ml	H001	-	Failed to form
9	10 Units/ml	4 mg/ml	150K/ml	H001	-	Failed to form
10	10 Units/ml	4 mg/ml	300K/ml	H001	72h	Electively sacrificed at 64d
11	10 Units/ml	4 mg/ml	300K/ml	H001	72h	Electively sacrificed at 64d
12	5 Units/ml	6 mg/ml	300K/ml	H002	48h	Ring Snapped at 5d
13	5 Units/ml	6 mg/ml	300K/ml	H002	48h	Ring Snapped at 6d
14	10 Units/ml	6 mg/ml	300K/ml	H002	24h	Electively sacrificed at 30d
15	10 Units/ml	6 mg/ml	300K/ml	H002	24h	Electively sacrificed at 30d
16	15 Units/ml	6 mg/ml	300K/ml	H002	24h	Electively sacrificed at 30d
17	15 Units/ml	6 mg/ml	300K/ml	H002	24h	Ring snapped at 8d
18	10 Units/ml	6 mg/ml	300K/ml	H001	24h	Electively sacrificed at 30d
19	10 Units/ml	6 mg/ml	300K/ml	H001	24h	Electively sacrificed at 30d
20	10 Units/ml	6 mg/ml	300K/ml	H001	24h	Ring snapped at 8d
21	10 Units/ml	6 mg/ml	300K/ml	H001	24h	Electively sacrificed at 30d
22	10 Units/ml	6 mg/ml	300K/ml	H001	24h	Electively sacrificed at 30d
23	10 Units/ml	6 mg/ml	300K/ml	H001	24h	Electively sacrificed at 30d
24	10 Units/ml	6 mg/ml	300K/ml	H001	24h	Ring snapped at 7d
25	10 Units/ml	6 mg/ml	300K/ml	H001	24h	Electively sacrificed at 30d
26	10 Units/ml	6 mg/ml	300K/ml	H001	24h	Ring snapped at 7d
27	10 Units/ml	6 mg/ml	300K/ml	H002	-	Failed to form by 14d
28	10 Units/ml	6 mg/ml	300K/ml	H002	24h	Electively sacrificed at 30d
29	10 Units/ml	6 mg/ml	300K/ml	H002	24h	Electively sacrificed at 30d
30	10 Units/ml	6 mg/ml	300K/ml	H002	24h	Electively sacrificed at 30d
31	10 Units/ml	6 mg/ml	300K/ml	H002	-	Failed to form by 14d
32	10 Units/ml	6 mg/ml	300K/ml	H001	24h	Electively sacrificed at 30d
33	10 Units/ml	6 mg/ml	300K/ml	H002	24h	Electively sacrificed at 30d
34	10 Units/ml	8 mg/ml	300K/ml	H002	48h	Electively sacrificed at 30d
35	10 Units/ml	8 mg/ml	300K/ml	H002	24h	Ring snapped at 4d
36	10 Units/ml	6 mg/ml	400K/ml	H002	24h	Ring snapped at 6d
37	10 Units/ml	6 mg/ml	400K/ml	H001	24h	Transplanted at 20d
38	10 Units/ml	6 mg/ml	400K/ml	H001	48h	Transplanted at 20d
39	10 Units/ml	6 mg/ml	400K/ml	H001	-	Failed to form by 14d
40	10 Units/ml	6 mg/ml	400K/ml	H001	24h	Transplanted at 20d
41	10 Units/ml	6 mg/ml	400K/ml	H002	-	Failed to form by 14 d
42	10 Units/ml	8 mg/ml	400K/ml	H005	24h	Rings snapped at 8d
43	10 Units/ml	8 mg/ml	400K/ml	H005	24h	Failed to form by 14d
44	10 Units/ml	8 mg/ml	400K/ml	H005	24h	Rings snapped at 6d

Table 5.1 Highlighted cells indicate HCSMMs which failed to form or lost structural integrity within 10d. Rows detail the concentration of thrombin/fibrinogen and seeding density of smooth muscle cells used for each HCSMM formed. Time taken to formation of a ring, length in culture and outcome for each model is also given.

5.2.2. Confirmation that fibrin-based HCSMMs contain smooth muscle

Although there was limited success in developing a HCSMM of reliable and consistent structural integrity, if a construct formed and remained intact by day 10, it could be maintained in culture for between 1 and 2 months, with all constructs being sacrificed electively (Table 5.1). Therefore it was of importance to ascertain whether each construct contained differentiated smooth muscle cells. HCSMMs were fixed with PFA at day 30 and either processed as paraffin sections for H&E or frozen sections to determine the expression of smooth muscle markers (Figure 5.2).

Mature (30d) HCSMMs were rarely uniform in their thickness (Figure 6.2A). Cross-sectional analysis demonstrated the presence of bands of cells surrounded by a significant layer of fibrin with little cellular infiltration (Figure 6.2B). Subsequent immunostaining for the expression of the smooth muscle markers desmin and smooth muscle actin (SMA) demonstrated dual labelling in the majority of cells within these bands, with no reactivity in the surrounding layers of fibrin (Figure 6.2C).

Figure 5.2 Confirmation that human smooth muscle cells can grow within fibrin-based HCSMMs

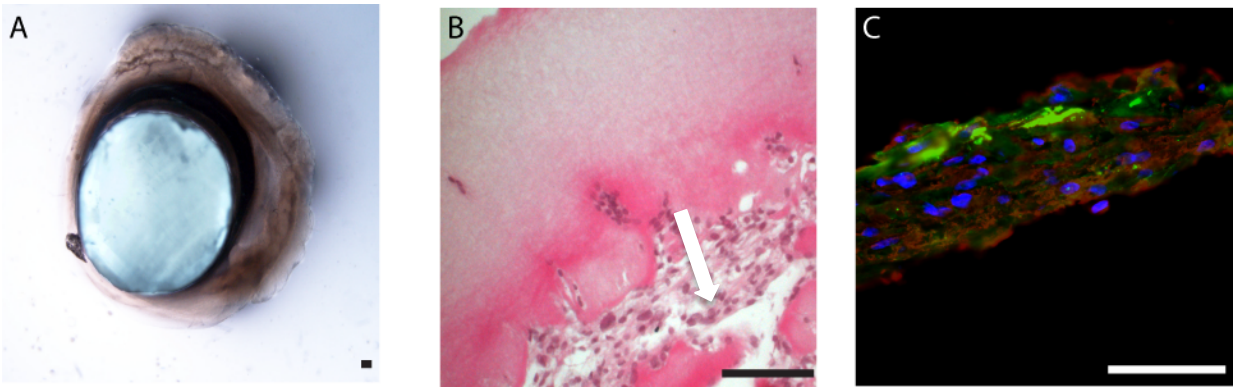


Figure 5.2. Mature HCSMMs were fixed with 4% PFA for 10minutes after 30d in culture (**A**). Each ring was bisected with half the ring embedded in paraffin for subsequent H&E analysis with the remaining half embedded for frozen sections. **B:** H&E staining demonstrates cells orientated in bands (white arrow) along the inner surface of each construct with very few cells infiltrating the outer fibrin layers. **C:** Standard immunofluorescence for the smooth human muscle markers smooth muscle actin (red) and desmin (green) reveals that the majority of cells express both of these common markers. Cell nuclei are stained with Dapi (blue). Scale bars = 50µm.

5.2.3. Transplanted ENSPC survive and migrate within fibrin-based HCSMMs

Having found that formed HCSMMs contain cells expressing typical markers for differentiated smooth muscle, the next question was to determine whether human ENSPC would survive and migrate within this environment.

To achieve this human ENSPC isolated from patients with HSCR were labelled with green fluorescent protein (GFP, see 2.6) and allowed to form neurospheres before being transplanted onto day 20 HCSMM (Figure 5.3). Live cell imaging of the constructs over the course of 7 days demonstrated that labelled ENSPC were still present in the HCSMM and suggested that cells were migrating away (50-100µm) from the transplantation site (Figure 5.3A-E).

The transplanted constructs were fixed at 10d and cross-sectional immunofluorescence imaging performed on frozen sections (Figure 5.3F,G). This confirmed that cells expressing GFP had both migrated into the constructs and also migrated away from the transplantation site (Figure 5.3F). On sections co-stained with the smooth muscle marker SMA, GFP+ve cells were identified in what appeared to be in a 'chain-like' arrangement, which may represent early neurite formation (Figure 5.3G).

Figure 5.3. Transplantation of GFP labelled human neurospheres onto HCSMMs

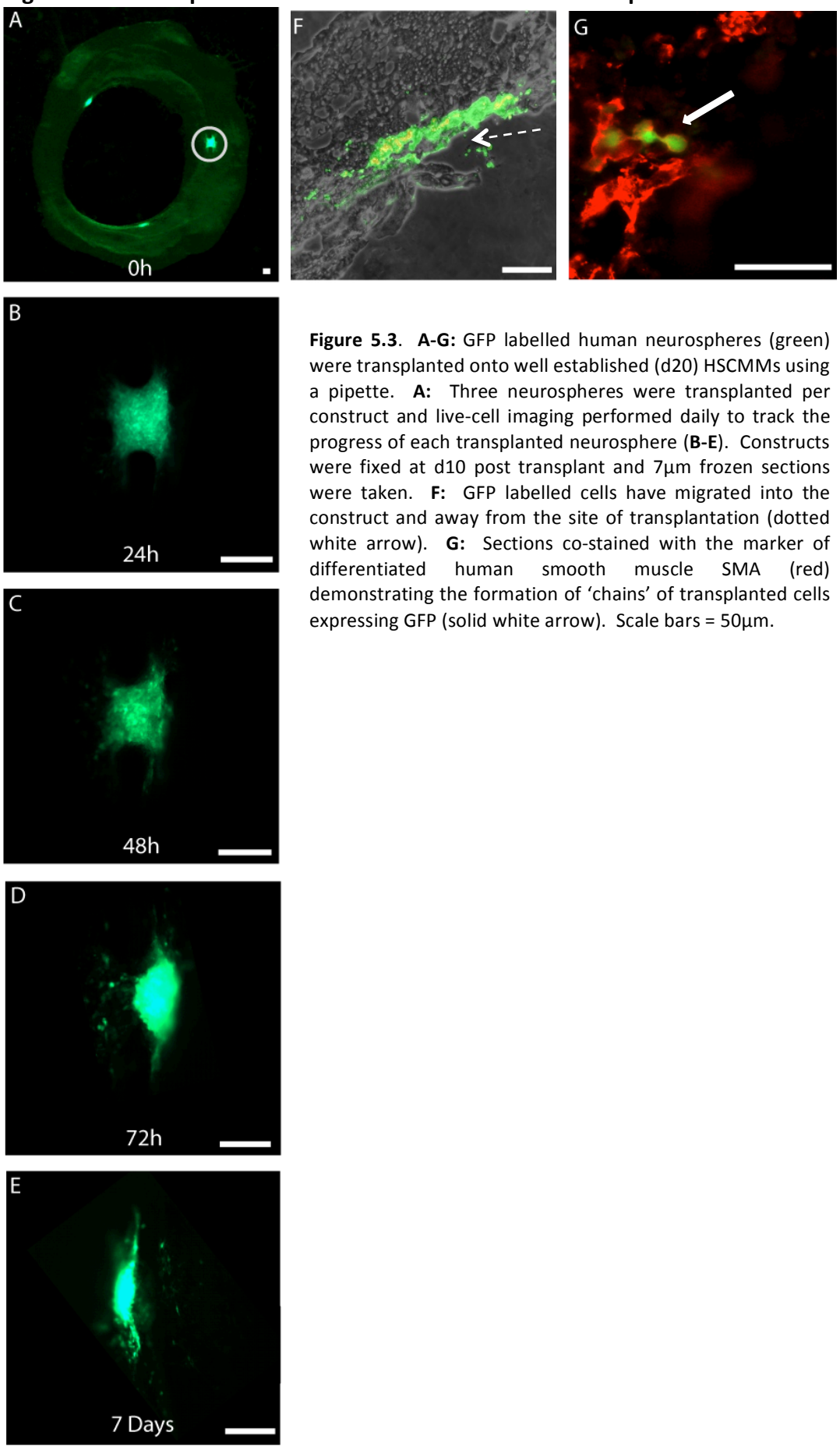


Figure 5.3. **A-G:** GFP labelled human neurospheres (green) were transplanted onto well established (d20) HSCMMs using a pipette. **A:** Three neurospheres were transplanted per construct and live-cell imaging performed daily to track the progress of each transplanted neurosphere (**B-E**). Constructs were fixed at d10 post transplant and 7µm frozen sections were taken. **F:** GFP labelled cells have migrated into the construct and away from the site of transplantation (dotted white arrow). **G:** Sections co-stained with the marker of differentiated human smooth muscle SMA (red) demonstrating the formation of 'chains' of transplanted cells expressing GFP (solid white arrow). Scale bars = 50µm.

5.2.4. Development of a decellularised mouse colon-based HCSMM

Although using the fibrin-based model permitted demonstration of the ability to grow human smooth muscle within the construct and confirmed that human ENSPC can grow within this environment, it was decided to look at alternative scaffolds for the HSCMM for two reasons: Firstly, the fibrin-based model was unreliable in forming a stable construct in culture and secondly, attempts to increase the number of smooth muscle cells seeded onto the construct reduced the reliability further. Following a wider review of the literature on the use of scaffolds in tissue engineering it was decided to follow the approach of researchers who had used a decellularised trachea as a scaffold (Baiguera*, et al.* 2010) and adapt this technique using a decellularised mouse colon as a scaffold and seeding it with human colonic smooth muscle.

To prepare the decellularised colonic scaffold colons were harvested from adult mice and the mesentery +/- any accompanying blood vessels stripped off before washing in distilled water (dH_2O). The scaffolds were then placed in a 1% (v/v) povidone-iodine solution (Sigma-Aldrich) for 30minutes, remove any bacterial contamination, followed by a further two washes with dH_2O. The decellularisation process was optimised to produce as scaffold that was microscopically free of cellular debris while maintaining its structural integrity (Figure 5.4). Each decellularisation cycle involved incubating the scaffolds in 4% sodium deoxycholate (SDC, Sigma-Aldrich) diluted in dH_2O for 24h on a mechanical rocker (Thermo-Scientific) at room temperature. On the second day, two further dH_2O washes were performed following a further 6h incubation with 2000KU DNase-I (Sigma-Aldrich) in 1M NaCl under the same conditions. Two further complete cycles were performed to remove all macroscopic traces of cellular material (see Figure 5.4). Once complete, scaffolds were stored in PBS containing 2% penicillin-streptomycin and 1µg/ml amphotericin B (Both Gibco) at 4˚C until required.

Figure 5.4. Optimisation of decellularisation protocols

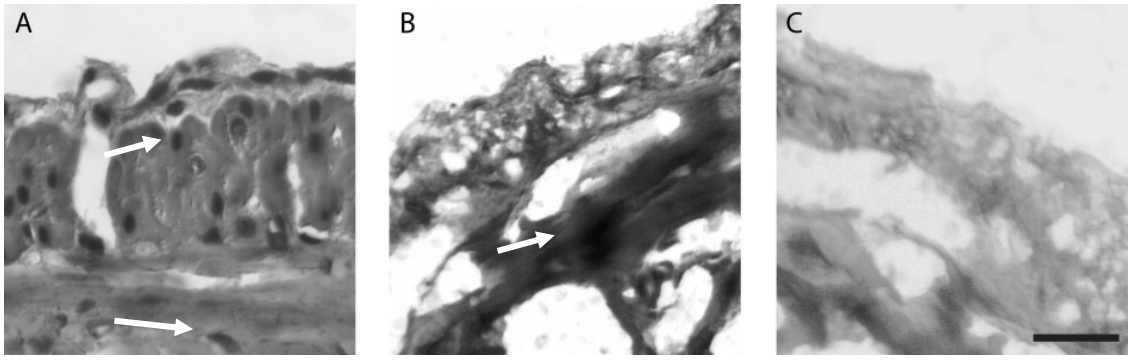


Figure 5.4. 7µm frozen sections of mouse colonic scaffolds stained with H&E to identify residual cellular material. **A:** Freshly isolated mouse colon prior to decellularisation, demonstrating the normal architecture of the colon, with the presence of distinct cells within the mucosa and muscle layers (arrows). **B:** Colonic scaffold after 1 cycle of decellularisation, demonstrating loss of the normal distinct architecture, but a significant amount of cellular/nuclear debris is still present (arrow). **C:** Scaffold after 3 complete cycles. The scaffold remains intact with minimal visible cellular debris remaining. Scale bar = 50µm.

The decellularised scaffold was then placed into a custom made InBreath® Bioreactor with a 3mm internal spindle (Harvard Apparatus, Kent, UK). The scaffold was secured to the spindle with ties before filling the bioreactor with FB media until the scaffold was fully covered (Figure 5.5). After a 1 h incubation period (37˚C, 5% CO_2) media was removed until the scaffold was exposed. Cell were then seeded directly onto the superior surface of the construct (see table 5.2) and returned to the incubator for 20 minutes before rotating the spindle 90˚. This process was repeated until cells had been seeded onto all four aspects of the scaffold. The construct was then returned to the incubator under the same conditions for 24h before adding fresh FB media to fully cover the construct and starting to rotate the spindle at 1.5 rpm. A second round of seeding was performed at 5 days if indicated in table 5.2.

Figure 5.5. Decellularised bowel model

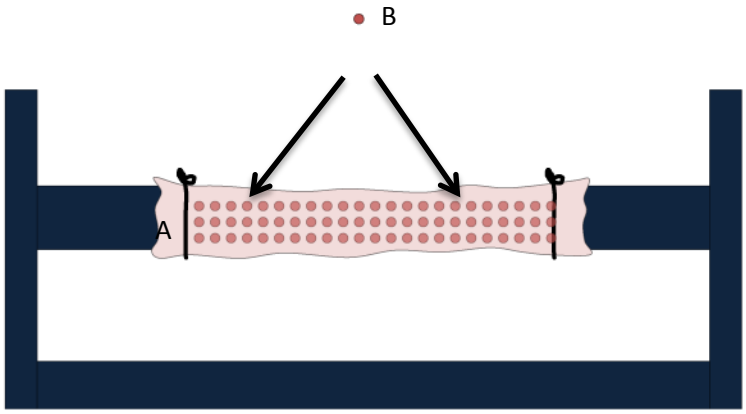


Figure 5.5. Diagram depicting the setup of the InBreath® bioreactor. The decellularised mouse colonic scaffold (**A**) is secured to the 3mm central spindles and the tissue bath filled with FB media to cover the scaffold. Following a 1h incubation (at 37˚C) media is removed to expose the scaffold and smooth muscle cells gently pipetted onto the superior surface. After 20mins the scaffold is rotated 90˚ and cells pipetted to the newly exposed surface. This process is repeated a further two times. The construct is then incubated for a further 24h under the same conditions before refilling with FB media to cover the scaffold and starting the bioreactor motor to continuously rotate the spindle at 1.5rpm.

Partial (50%) media changes are made every 4 days and as with the fibrin-based model the media was changed to HS medium after 1 week to stimulate the differentiation of mature smooth muscle cells.

Each decellularised colon HCSMM formed is recorded in table 5.2. The most striking difference between the models is that all but one construct remained stable until the end of the experiment and the only failure was due to infection rather than mechanical instability. Constructs remained stable whether one or two seeding cycles were performed.

Table 5.2 List of conditions and outcomes for each decellularised colon HCSMM

HCSMM Number	Cell Seeding Density	Number of seeding cycles	Outcome
45	1M/ml	1	Electively sacrificed at 30d
46	1M/ml	1	Electively sacrificed at 60d
47	1M/ml	2	Electively sacrificed at 30d
48	1M/ml	2	Electively sacrificed at 60d
49	2M/ml	2	Infected at 28 days
50	1M/ml	2	Physiological testing at 30d
51	1M/ml	2	Physiological testing at 30d

Table 5.2. Highlighted cell indicates a construct lost prior to completion of the experiment due to infection at 28 days. Rows detail the cell seeding density of human aganglionic colonic smooth muscle cells used for each HCSMM formed. The number of seeding cycles, the length of time in culture and outcome for each model is also given.

5.2.4. Decellularised colonic HCSMM contains physiologically active smooth muscle

Although the decellularised colon-based HCSMMs solved both the structural reliability issues associated with the fibrin-based models and allowed repeated cycles of cell seeding, it was still to left to be determined whether the seeded aganglionic colonic smooth muscle cells would survive and integrate with the scaffold itself. Hence, 30d old constructs (45 and 47, see table 5.2) were fixed with 4% PFA and frozen sections prepared to determine the expression of markers of mature human smooth muscle. Figure 5.6 shows that after 30d in culture HCSMMs contain cells expressing the mature human smooth muscle marker SMA.

Figure 5.6. Seeded HCSMMs contain cells expressing smooth muscle markers

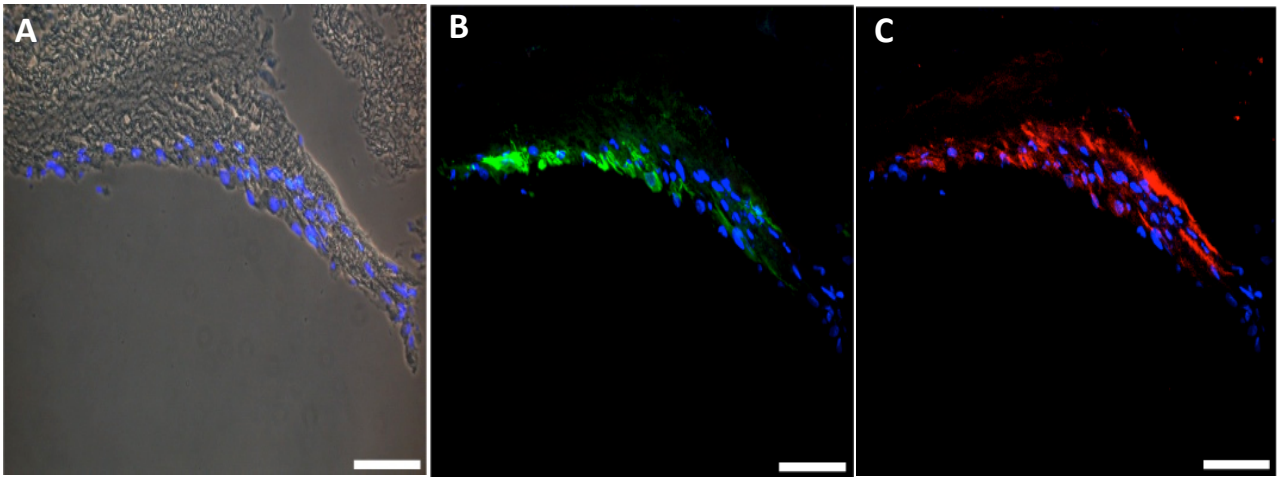


Figure 5.6. Aganglionic human colonic smooth muscle was seeded onto a decellularised mouse scaffold and cultured for 60d. The constructs were fixed with 4% PFA and 7µm frozen sections prepared for analysis. **A:** Cell nuclei (stained with dapi – blue) can be seen lining the surface of the construct and are found to have migrated several cells deep into the construct. **B-C:** These cells were found to express typical markers of mature human colonic smooth muscle: desmin (green) and smooth muscle actin (red). Scale bars = 50µm.

Electrophysiological assessment of the same constructs was performed to determine whether they had contractile properties consistent with colonic smooth muscle. Both HCSMMs generated contractions with forces of up to 150µN in response to both KCl and carbachol (Figure 5.7). Furthermore, the biphasic response seen following stimulation with carbachol is similar that seen in other studies using colonic smooth muscle (McCarron*, et al.* 2002).

Figure 5.7. HCSMMs exhibit a physiological response consistent with smooth muscle

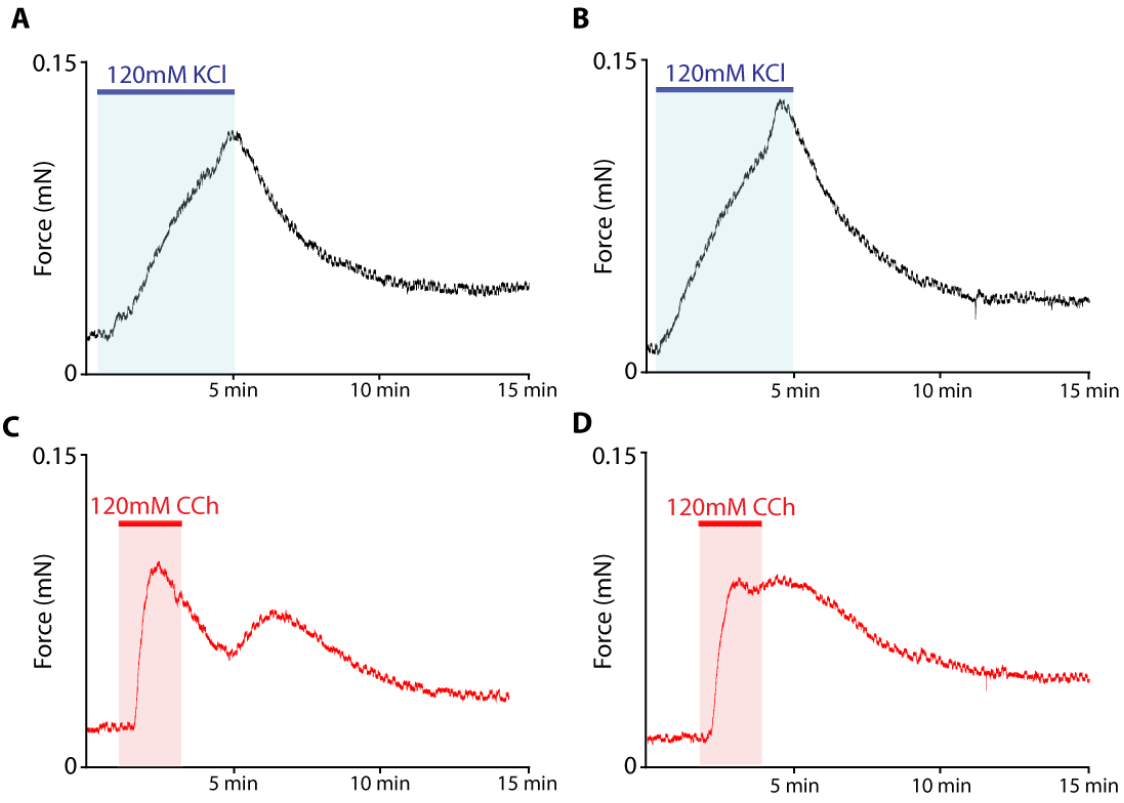


Figure 5.7. Two 30d old HCSMM were cut into 1cm strips for electrophysiological analysis. Strips were attached to a force transducer and buffered in physiological saline at 37˚C before determining their response to 120mM KCl and 120mM carbachol. Typical force/time traces are demonstrated in **A-D**. A-B: Both constructs had a positive response to KCl infusion generating contractions with forces of >100µN. C-D: Similarly, infusion of carbachol resulted in a swift contraction and slow relaxation phase.

5.2.5. Cultured smooth muscle from aganglionic human bowel contains cells with a neuronal phenotype

Human aganglionic smooth muscle was maintained in culture for periods of up to 12 weeks prior to use in in the HCSMM. During routine passaging of these cell cultures it was noted that they contained cells with a neuronal phenotype (figure 5.8) and within one culture flask appeared to form spheroid neurosphere-like bodies. This unexpected finding led to the further studies described in chapter 6 and meant that no further work was undertaken on the HCSMM during my remaining time in the lab.

Figure 5.8. Cells with a neuronal phenotype can be identified in aganglionic smooth muscle cultures.

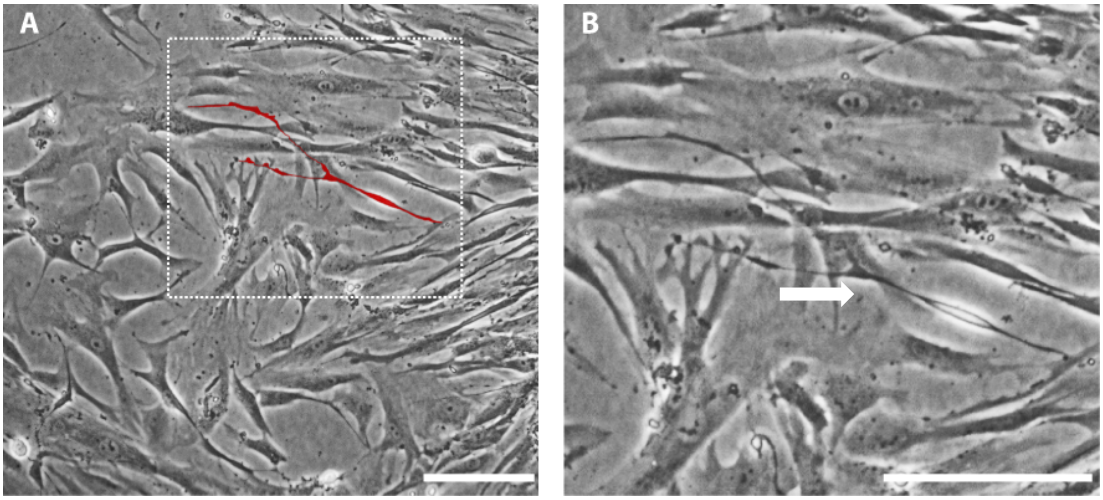


Figure 5.8. 14d old human aganglionic colonic smooth muscle cultures maintained under standard conditions. **A:** Cells with a neuronal phenotype were identified within cultures derived (highlighted in red). **B:** Higher power image of region of interest in **A**. White arrow demonstrates the presence of a cell with a neuronal phenotype. Scale bars = 50µm.

5.3. Discussion

Previous work has highlighted the therapeutic potential of autologous ENSPC transplantation for HSCR (Dong*, et al* 2008, Lindley*, et al* 2008, Hotta*, et al* 2013). In order to progress towards human clinical trials we need to be able to predict how these rapidly proliferating cells will behave once transplanted into human colonic smooth muscle. This chapter describes the development of a human colonic smooth muscle model (HCSMM) that is derived from the aganglionic colon of patients with HSCR. It demonstrates aganglionic human colonic smooth muscle can be maintained in culture on a scaffold and more importantly that these cells retain both the typical expression profile and electrophysiological responses of human colonic smooth muscle. Thereby suggesting that models such as these have the potential advantage of allowing us to investigate the behaviour of transplanted ENSPC in an environment that is closer to that of the human colon.

5.3.1 Development of a fibrin based HCSMM

The original protocol, that the fibrin-based HCSMM was based on, was developed with the intention of developing an artificial internal sphincter (Hecker 2005, Somara*, et al* 2009). This had the attraction of being a cheap and potentially simple scaffold on which to seed aganglionic colonic smooth muscle that could be maintained in culture for a significant length of time. The results within this chapter show that a fibrin-based HCSMM can meet two of the main aims of this chapter: Firstly, that the model could be maintained in culture for at least 2 weeks (up to 70 days in some cases), and secondly that these cells organised themselves into bands similar to and retaining the typical expression profiles of human colonic smooth muscle. Furthermore, the important finding that human ENSPC can be transplanted into the model and migrate within it forming ‘chains-like’ arrangements

confirms, in principle the potential for a HCSMM to enable us to observe ENSPC and their interactions with human colonic smooth muscle.

However, significant issues lie with the remaining two aims, which are both linked together: the ability to allow physiological testing pre and post ENSPC transplantation and the reproducibility (at best 68%). The original reports of the forces generated by this model in the literature are only in the range of 10-20μN (Somara*, et al* 2009), which although they confirm the presence of physiologically active smooth muscle within these models the forces are of such low levels that it would be difficult if not impossible to reliably determine the effect of ENSPC transplantation. Attempts were made to increase the seeding density of smooth muscle cells with the aim of increasing the forces generated, however under these conditions the failure rate rose to 66% with the rings typically breaking within the first week of seeding.

Because the majority of failures of the fibrin-based HCSMM were due to problems with the fibrin scaffold failing to form or 'snapping' there was a need to look for alternative scaffolds. For its originally intended purpose using a fibrin-based scaffold had the major advantages that it would not stimulate an immune response when transplanted, it would be biodegradable and that it is a simple and cheap solution. However, these were not key factors in the development of a HCSMM as there was no intention to transplant the model into an in-vivo environment. Therefore, alternative scaffolds were sought that might meet all of the required aims.

5.3.2. Development of a decellularised mouse colon based HCSMM

As previously stated the difficulty with observing the behaviour of ENSPC transplanted into colonic explants is maintaining the explant in culture long enough to observe the effects of the ENSPC on the host environment. As this is predominantly an issue of diffusion of oxygen and nutrients (Griffith*, et al* 2005), which in itself is related to the thickness of the explant, initial attempts were made to simply shave a thin layer of smooth muscle from colonic biopsies and maintain this in culture. However, it was not was not possible to do this. There was inconsistency in the thickness of samples, leading to some dying within 24h in cultures while the thinner sections would fail to maintain their structure. This prompted the search for a more robust scaffold on which smooth muscle cells could be seeded upon.

The majority of current work on in-vitro smooth muscle engineering has the aim of developing a graft or vessel replacement for vascular surgery, although there are groups looking to develop a tissue engineered colon replacement (Denost*, et al.* 2013, Y. Tan*, et al.* 2014, Walthers*, et al.* 2014). However, the majority of these methods are based on implanting a scaffold into the colon and allowing cells to migrate onto and colonise the graft using both artificial and biological scaffolds (Henne-Bruns*, et al.* 1990, Oh*, et al.* 2002, Hoeppner*, et al.* 2009, Nocca*, et al.* 2009). Because aim of this chapter was to develop a model that could be maintained in-vitro these methods were not applicable, instead a technique used to transplant a cadaveric trachea implanted with autologous stem cells was taken as starting point (Macchiarini*, et al.* 2008, Baiguera*, et al* 2010). The decision to utilise mouse colon rather than tracheal cartilage as a scaffold was taken to allow the model to contract and also to reduce the size of the construct making it easier to maintain in culture.

Because there was no intention to transplant the resulting HCSMM, there was no requirement for the scaffold to lack any immunogenicity. Therefore, after optimisation the

decellularisation process was found to be much shorter than that required for trachea as the aim was limited to produce a microscopically decellularised scaffold for ex-vivo analysis.

The decellularised mouse scaffold we developed maintained cells expressing typical colonic smooth muscle markers in long-term culture, was reproducible and importantly had the characteristic electrophysiological response of colonic smooth muscle (McCarron*, et al* 2002). Furthermore, the forces generated by the decellularised colon-based HCSMM are up to 15x greater than those published using the fibrin based model (Somara*, et al* 2009). This would potentially make it easier to identify changes in contractility following transplantation of ENSPC. Taken together, these results suggest that the decellularised HCSMM has the potential to be a useful tool with which to observe the behaviour of transplanted ENSPC in the microenvironment of mature human colonic smooth muscle. This may enable us to more accurately predict how these cells might behave post transplantation, and in particular demonstrate if they continue to proliferate or alternatively return to an essentially quiescent state as they were prior to isolation. The model does not provide a perfect replica of the colonic microenvironment, but at present it is not possible to replicate this within the lab, however it does help provide an environment that can act as a stepping-stone between animal studies and human clinical trials.

The major practical limitation of this model is the requirement for the bioreactor, meaning that with only one available in the lab new constructs can only be made in series rather than parallel. The implication of this is that it limits the speed at which the production of the HCSMM can be optimised. The results in the chapter, although encouraging still leave a number of steps that need to be completed before the model could be used reliably. Cell seeding density and time in culture need to be optimised according to the electrophysiological response that they generate. The inter-construct reliability needs to

be determined, for example, does the electrophysiological response between smooth muscle cells derived from different patients?

Although much work still remains to be done, these preliminary results suggest that a HCSMM, based on a decellularised mouse colonic scaffold has the potential to provide valuable information on ENSPC behaviour prior to progressing towards human clinical trials.

5.3.3. Cultured smooth muscle contains cells with a neuronal phenotype

The observation that cells with a neuronal phenotype are present within aganglionic smooth muscle cultures was surprising. Initially it was thought that the colonic biopsies must have been taken in the transitional zone of the colon and the cells seen were a contaminant from the more proximal ganglionic bowel. However, this is improbable as the neuron-like cells were found in cultures derived from nine patients all of which had been determined by paediatric histopathologists to be well below the transitional zones. By definition the aganglionic bowel should not contain neuronal cells, because although abnormally thickened extrinsic nerve fibres are found in the aganglionic colon their cell bodies are found in the pelvic ganglia (Tam*, et al.* 1990).

This finding raises a number of questions, such as: Where are these cells coming from? What are they? Are they functional and do they have any therapeutic potential for the treatment of HSCR? These questions formed the basis of the work described in chapter 6.

5.4. Conclusions

The results in this chapter have shown that it is possible to culture aganglionic human colonic smooth muscle on biological scaffolds. Furthermore, the resulting HCSMMs can reliably be maintained in culture for more than 2 weeks, and can undergo physiological testing, enabling a comparison to be made both pre and post transplantation with human ENSPC. This would allow further study of ENSPC behaviour, helping develop our understanding of their control mechanisms and helping us to more accurately predict how they might behave once transplanted into the human colon.

However, perhaps of greater significance is the incidental observation that cells with a neuronal phenotype are present within human aganglionic smooth muscle cultures. This raises several important questions such as: as to are these cells indeed neuronal, what is their origin, are they functional and do they have the potential to be used as part of any future therapy.

Chapter 6: Neurospheres from Aganglionic Hirschsprung Bowel

6.1. Introduction

6.1.1. Background

The presence of ENSPC within normal ganglionic bowel is widely reported (Almond*, et al* 2007, Lindley*, et al* 2008, Metzger*, et al* 2009), but despite on-going study into their therapeutic potential for conditions such as HSCR the location of these progenitor cells within the ganglionic bowel has yet to be determined. Recent studies have sought to identify the source of neural progenitors within the bowel have suggested a glial origin, supported by the expression of glial markers (GFAP, Sox10, S100) and lineage tracing studies (Joseph*, et al* 2011, Laranjeira*, et al* 2011). However, the lack of a definitive marker for ENS neural progenitors has hampered the study of their location and behaviour in-vivo. There has been a presumption that these progenitors arise from cells populating the bowel during the initial craniocaudal migration of enteric NCCs during development, with studies suggesting that they are located in the region of the myenteric plexus within enteric ganglia (Kruger*, et al* 2002), thereby explaining their absence from distal aganglionic bowel seen in HSCR.

The incidental observation that cells with a neuronal phenotype are present within in-vitro cultures of human aganglionic smooth muscle (see chapter 5) raises significant questions regarding these existing presumptions. There has been limited study into the properties of aganglionic bowel, and what work has been carried out has been largely limited to descriptive studies. It is well documented that the aganglionic regions of colon found in

HSCR do not contain components of the intrinsic nervous supply to the bowel but do contain abnormally thickened nerve trunks traversing through them in a largely disorganised arrangement (Baumgarten*, et al.* 1973, Tam*, et al* 1990). No previous study has looked for the presence of ENSPC within the aganglionic bowel.

6.1.2. Aims

The identification of cells with a neuronal phenotype present within aganglionic smooth muscle cultures raises significant questions regarding our current understanding of the source of neural progenitors within the bowel. Therefore, the aim of the work in this chapter was to: Firstly, determine whether these cells previously identified were indeed neural progenitors; Secondly, to make a comparison between progenitors derived from both the normal ganglionic and the aganglionic bowel from patients with HSCR; and finally to make an assessment of the functional potential of these cells, i.e. did they have any therapeutic potential for the treatment of HSCR?

6.2. Results

6.2.1. Cells from human aganglionic colonic biopsies have the potential to form neurospheres in-vitro.

The presence or absence of enteric ganglia was confirmed both as part of the routine histopathological diagnosis by clinical pathologists and within the lab (Figure 6.1). Thickened extrinsic nerve fibres were also identified in both the submucosal and myenteric plexi of aganglionic biopsies. Of note, a striking difference in the localisation of expression for the neural crest marker P75 between human aganglionic and ganglionic biopsies was seen in the submucosal plexus. Consistent with previous observations (Kobayashi*, et al.* 1994), nerve fibres within aganglionic colon were found to express P75 throughout with a predominantly peripheral pattern of localisation around the epineurium. However, the weaker staining seen within the endoneurium is similar in both ganglionic and aganglionic fibres, consistent with the expected distribution of Schwann cells (Figure 6.1 E-H).

To assess the potential of cells derived from human aganglionic biopsies to develop a neuronal phenotype, primary aganglionic smooth muscle cells were cultured in media supplemented with horse serum in order to promote differentiation (Figure 6.1 G-I). After 48 hours, cell bodies became compact rather than spread on the substrate, cells started to develop multiple projections before beginning to form aggregates. After seven days in culture these aggregates had formed neurosphere-like bodies, which were identical to the macroscopic appearance of neurospheres derived from human ganglionic colonic biopsies and grown under standard conditions.

Human aganglionic cultures maintained under conditions to promote growth (see 2.3.2.2) were found to contain a high proportion of cells expressing the smooth muscle marker SMA (Figure 6.2B), with fewer cells expressing the neural crest marker P75 (Figure 6.2A). There

was no apparent expression of either the glial marker GFAP or the mature neuronal marker PGP9.5 (Figure 6.2C-D). In contrast, analysis of neurosphere-like bodies formed from the same patient under conditions promoting differentiation identified an increased number of cells expressing P75 (Figure 6.2E). Alongside this there was a reduction in the proportion of cells expressing smooth muscle markers (Figure 6.2F) and the appearance of cells expressing both glial and neuronal markers (Figure 6.2 G-H).

The next step was to determine whether these aganglionic-derived neurosphere-like bodies are inherently different to those derived using established techniques from ganglionic human colon (Almond*, et al* 2007, Lindley*, et al* 2008). Therefore, neurospheres were generated from the ganglionic colon from the same patient and comparison of the expression of typical neural crest, glial and neuronal markers was made. Importantly, there was no apparent difference in either the pattern of expression or cell phenotype between cells derived from the aganglionic- or ganglionic-derived neurospheres (Figure 6.2 I-L). This prompted a more detailed analysis of the development of the expression of typical ENS markers.

Figure 6.1. Aganglionic HSCR colon contains cells that can form neurosphere-like

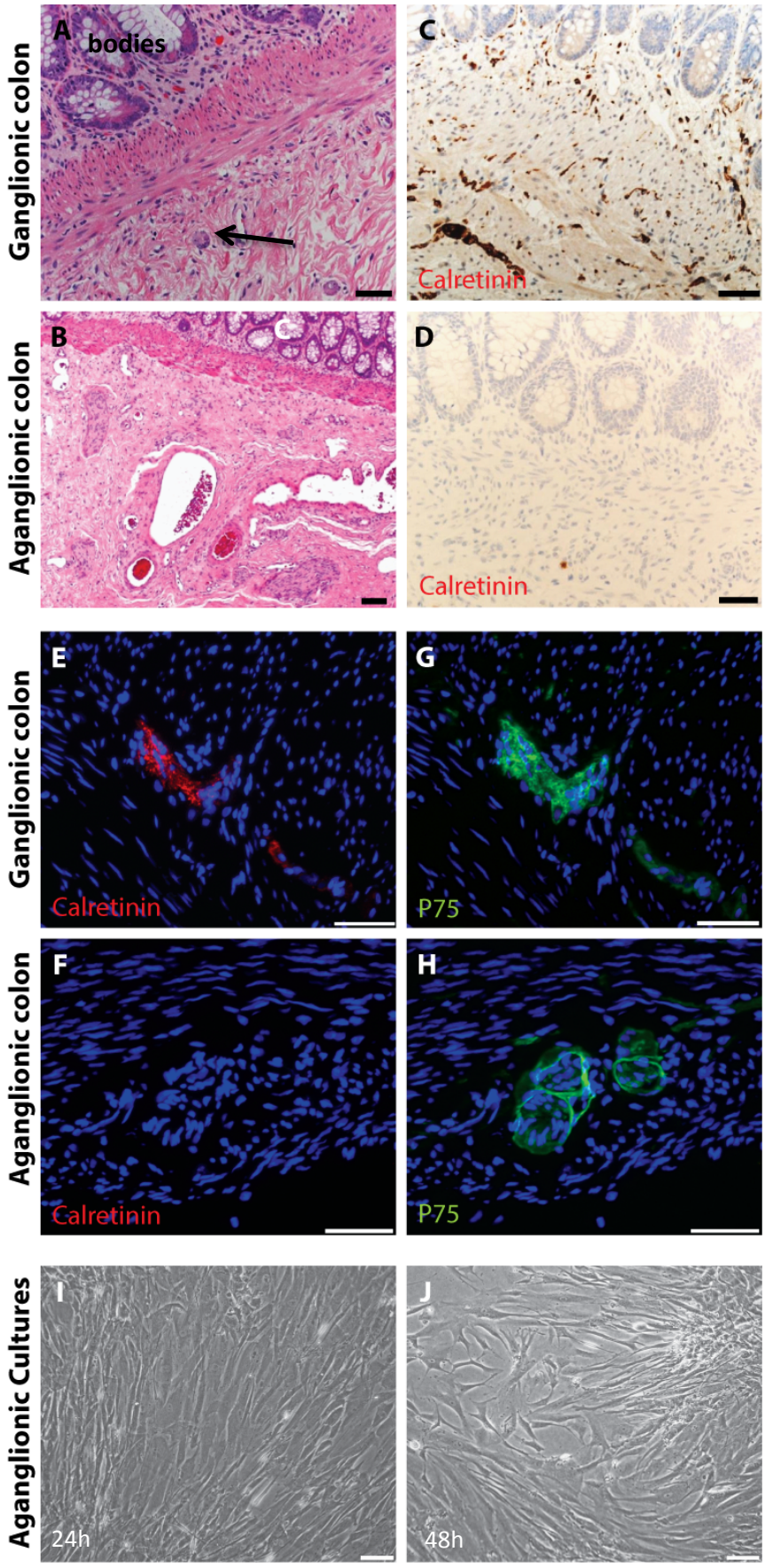


Figure 6.1. A-D: 4µm paraffin sections taken from ganglionic and aganglionic colonic biopsies taken from the same patient. **A-B:** The absence of enteric ganglia (black arrow) was confirmed on H&E analysis. **C-D**: Calretinin expression (brown) is seen in ganglionic sections but not aganglionic. **E-F:** 7µm frozen sections confirm the presence of calretinin expression (red) in ganglionic sections (**E**) and absence in aganglionic sections (**F**). **G-H:** Labelling with the neural crest marker P75 (green) revealed a marked difference in the localisation of P75 aganglionic sections (**G-H**), with prominent expression of P75 in the perineurium of extrinsic nerve fibres (**H**). Nuclei are counterstained with dapi (blue).

I-K: Phase contrast microscopy of aganglionic smooth muscle cultures. **I**: Smooth muscle cultured under standard conditions. **J:** 48h after addition of HS media to promote differentiation cells aggregate together, demonstrating a more elongated, neuronal phenotype with multiple projections. Cells continued to aggregate over time and by 7days (**K**) neurosphere-like bodies were seen. *Scale bars* =100μm **A-H**, and 50μm **I-K**.

6.2.2. HSCR aganglionic-derived neurospheres possess increasing proportions of cells expressing neural crest markers

Unsurprisingly, when isolated from fresh human colonic tissues the majority of cells in the initial isolate expressed the smooth muscle marker smooth muscle actin (SMA, Figure 6.3). After 10 days in culture the proportion of cells expressing SMA dropped significantly in the first generation of neurospheres formed, and remained low over subsequent generations. No significant difference was seen between neurospheres derived from human aganglionic or ganglionic colonic biopsies. In contrast the proportion of cells expressing the neural crest marker P75 was low in both the initial human aganglionic and ganglionic cell isolates but rose quickly with approximately one third of cells expressing P75 in first generation neurospheres (Figure 6.3). Over the following 20 days this trend continued with the majority of cells derived from tertiary neurospheres expressing the neural crest marker at 30 days.

Figure 6.2. HSCR aganglionic-derived neurospheres contain cells similar to those derived from ganglionic colon

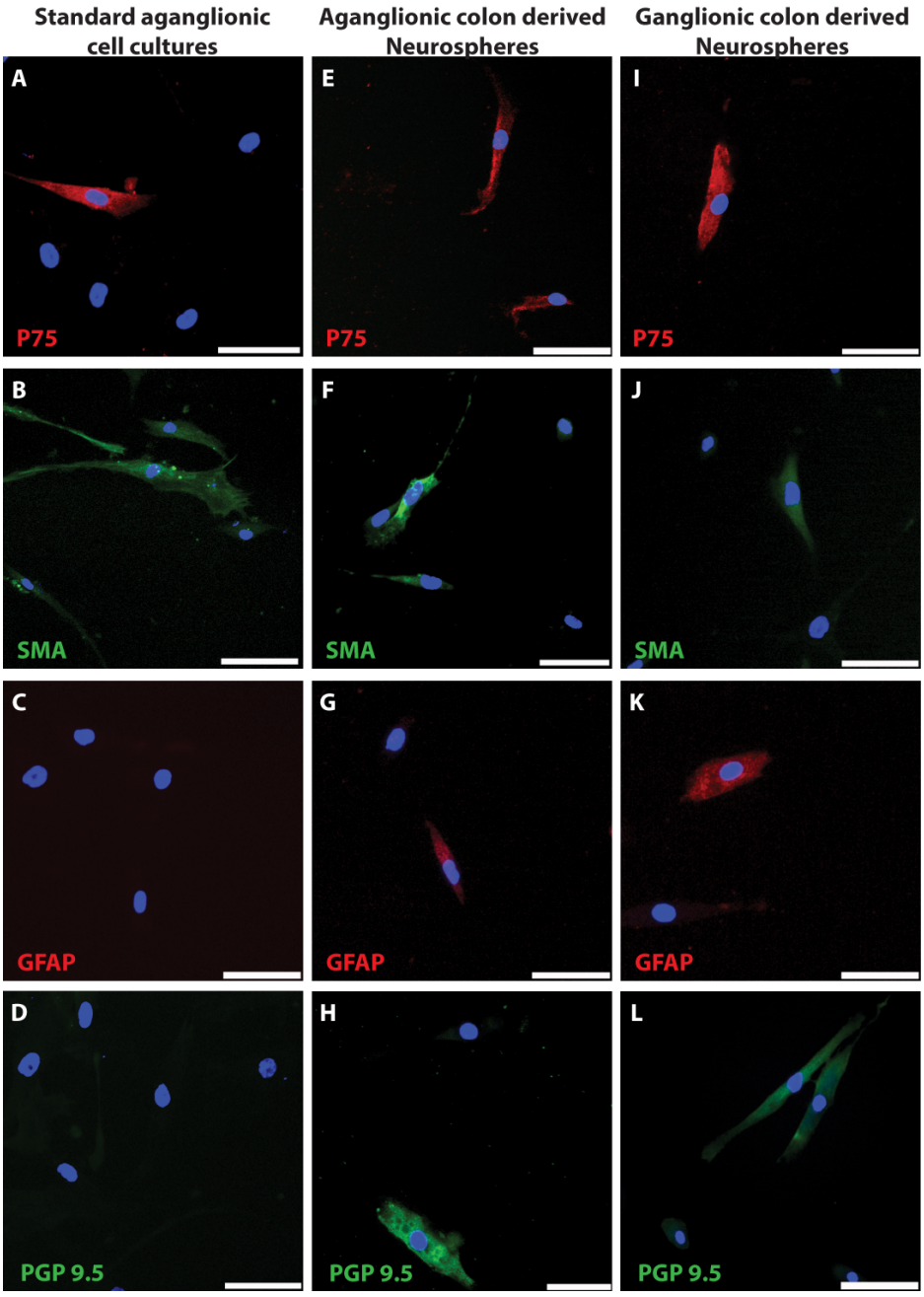


Figure 6.2. Both primary cultures of human aganglionic smooth muscle (**A-D**) and 14d old neurospheres (**E-L**) were dissociated and seeded onto fibronectin coated chamber slides before fixing and labelling at 24h. All samples were taken from the same patient. Cells were stained for expression of the neural crest marker P75 (red), smooth muscle actin (SMA, green), the glial marker GFAP (red) and the mature pan-neuronal marker PGP9.5 (green). Dapi (blue) was used to counterstain cell nuclei. **A-D:** The majority of cells from smooth muscle cultures typically express SMA (**B**), a smaller number of cells express P75 (**A**) and there is no significant expression of glial and neuronal markers (**C-D**). In contrast, cells from human aganglionic-derived neurospheres (**E-H**) and ganglionic-derived neurospheres (**I-L**) are shown to express both glial and neuronal markers (**G-H, K,L**). Furthermore, an increased proportion of cells were found to express P75 alongside a corresponding fall in the number of SMA positive cells (***E*-F, I-J**). Scale bars = 50µm.

Figure 6.3. Expression of smooth muscle and neural crest markers over time in human HSCR aganglionic- and ganglionic-derived neurospheres

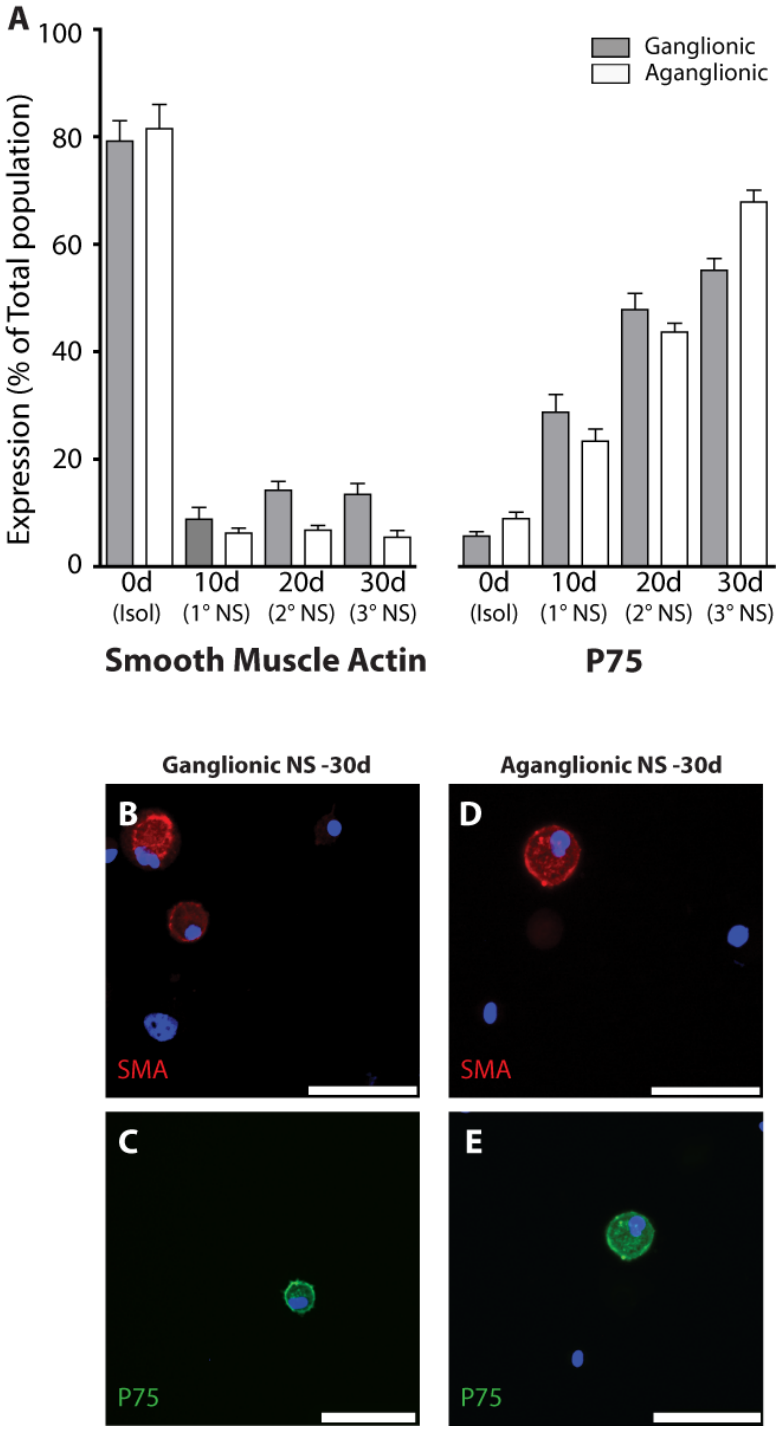


Figure 6.3. Expression of the smooth muscle marker smooth muscle actin(red) and neural crest marker P75 (green) in cells derived from both ganglionic and aganglionic human colon over time. Dapi (blue) was used to counterstain cell nuclei. Cells were analysed at isolation and follow routine passaging at 10d intervals. The proportion of cells expressing smooth muscle markers (**A**) drops significantly after isolation (0d) and remains low in subsequent neurosphere generations. Expression of P75 (**A**) is low in initial isolates (0d) but increases over time until the majority of cells are P75 positive at 30 days. No significant differences were identified between cells derived from ganglionic and aganglionic sources (**A-E**). Scale bars = 50µm, Error bars represent standard error of the mean (SEM) from 5 experiments.

6.2.3. HSCR aganglionic-derived neurospheres possess increasing proportions of the immature glial marker GFAP over time.

As seen with the neuronal markers, the proportion of cells expressing of the immature glial marker glial fibrillary acidic protein (GFAP) increased significantly during the maturation of neurospheres with nearly 50% of cells expressing GFAP in tertiary neurospheres at 30 days (Figure 6.4) . However, this was not seen in the mature glial/Schwann cell marker S100; expression of S100 in the initial single cell isolates was more than ten times higher than that seen for the immature marker GFAP. The proportion of cells expressing S100 increased in 1st generation neurospheres and subsequently gradually decreased, however this was not statistically significant. No significant difference was identified between human aganglionic and ganglionic derived cells.

6.2.4. HSCR aganglionic-derived neurospheres possess increasing proportions of cells expressing neuronal markers over time

At the start of culture, expression of the pan-neuronal marker PGP9.5 was limited to a small minority of cells (Figure 6.5). This proportion increased significantly with time in culture with more than 40% of cells expressing the neuronal marker in mature (30 day old) tertiary neurospheres. Dual labelling with the ENS specific marker calretinin was absent in cells isolated from aganglionic colon, but importantly after 10 days in culture approximately 10 % of cells were identified as calretinin positive in both aganglionic and ganglionic derived neurospheres. The proportion of positive cells continued to increase with time in culture, however this increase was significantly smaller in the aganglionic-derived neurospheres. 18% cells in tertiary aganglionic-derived neurospheres expressed calretinin vs. 30% of cells in tertiary ganglionic-derived neurospheres at day 30.

Figure 6.4. Expression of glial markers over time in human HSCR aganglionic- and ganglionic-derived neurospheres

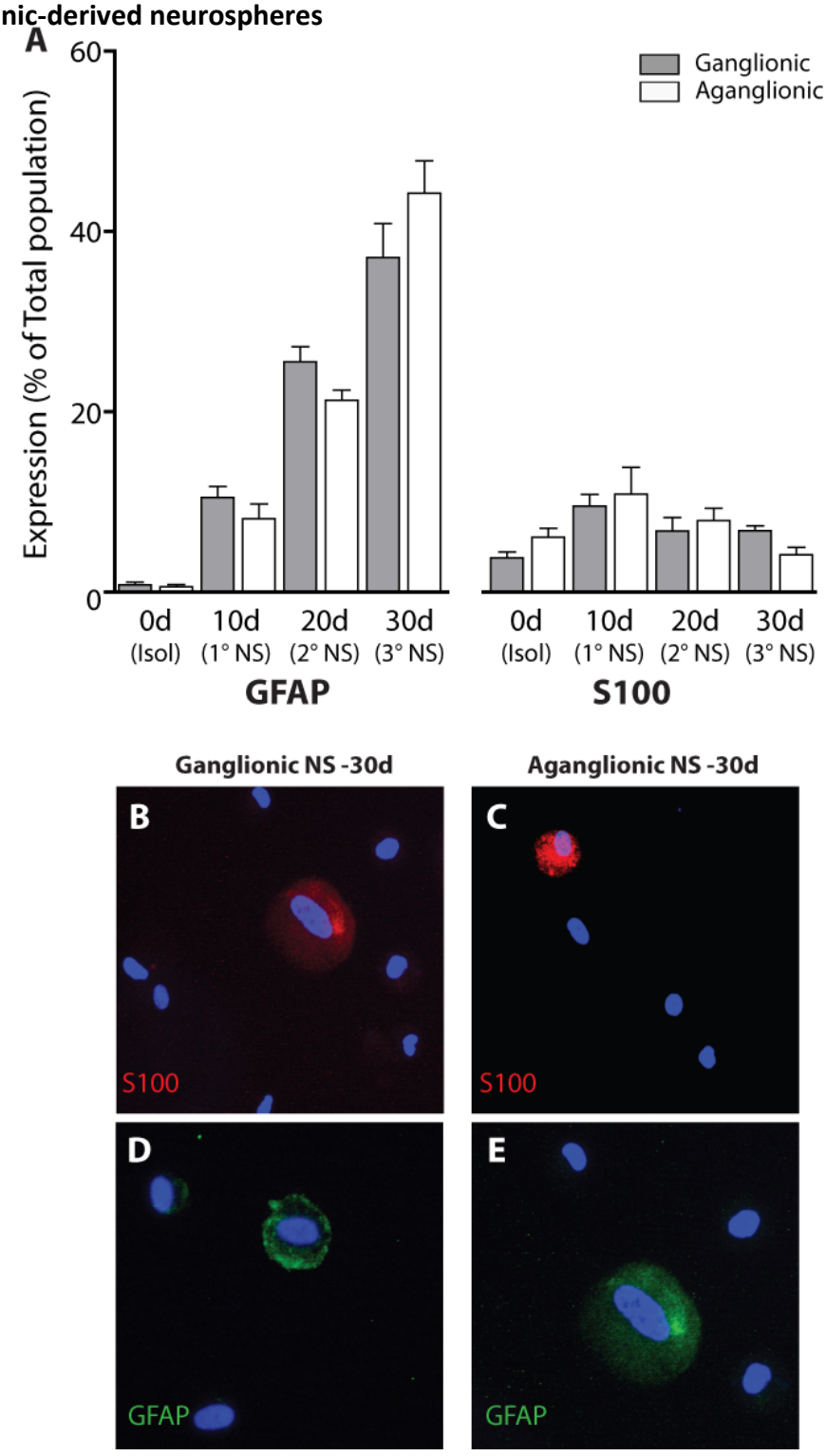


Figure 6.4. Expression of the GFAP (green) and S100 (red) glial markers in cells derived from both ganglionic and aganglionic human colon over time. Dapi (blue) was used to counterstain cell nuclei. Cells were analysed at isolation and follow routine passaging at 10d intervals. In the initial isolate there are minimal cells expressing GFAP, however this proportion increases steadily over time (**A**). S100 is expressed in freshly isolated cells, however after an initial rise it gradually falls over time (**A**). No significant differences (P>0.05) were identified between cells derived from ganglionic and aganglionic sources (**A-E**). Scale bars = 50µm, Error bars represent standard error of the mean (SEM) from 5 experiments.

Figure 6.5. Expression of neuronal markers over time in human HSCR aganglionic- and ganglionic-derived neurospheres

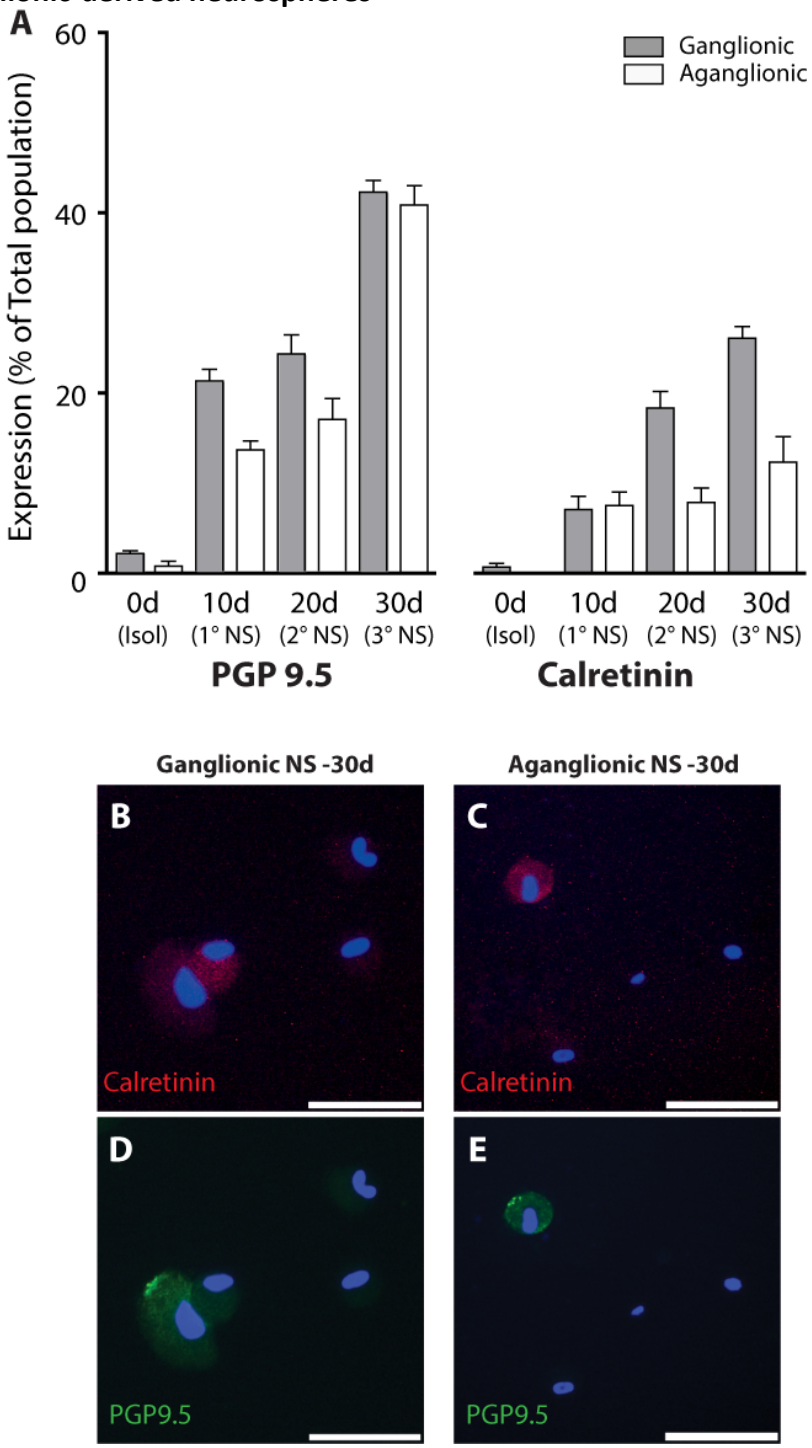


Figure 6.5. Expression of the pan-neuronal marker PGP9.5 (green) and the ENS specific marker calretinin (red) in cells derived from both ganglionic and aganglionic human colon over time. Dapi (blue) was used to counterstain cell nuclei. Cells were analysed at isolation and follow routine passaging at 10d intervals Expression of the pan-neuronal marker PGP9.5 increases steadily over time with no significant difference seen between ganglionic and aganglionic samples (**A, D, E**). No expression of calretinin was detected in initial aganglionic isolates, by 10d cells expressing this ENS specific marker can be identified and by 30d more than 10% of cells in mature (3º) neurospheres are positive (**A, B,C**). However, the proportion of calretinin+ cells remains significantly lower ($P<0.001$) than that seen in human ganglionic derived neurospheres. Scale bars = 50µm, Error bars represent standard error of the mean (SEM) from 5 experiments.

6.2.5. HSCR aganglionic-derived neurospheres contain cells capable of differentiating into mature neuronal phenotypes

To determine whether human aganglionic-derived neurospheres contain cells with the potential to differentiate into mature neuronal phenotypes typically seen within the ENS neurospheres were plated out on poly-D-lysine/laminin coated dishes. At day 7 cells with a distinct neuronal morphology were seen that expressed the mature neuronal markers nNOS and VIP. In addition cells were also identified expressing both the immature neuronal marker Tuj and calretinin, which is only found within ENS neurons and not those of an extrinsic origin.

Figure 6.6. Aganglionic-derived neurospheres can differentiate into cells expressing mature neuronal phenotypes

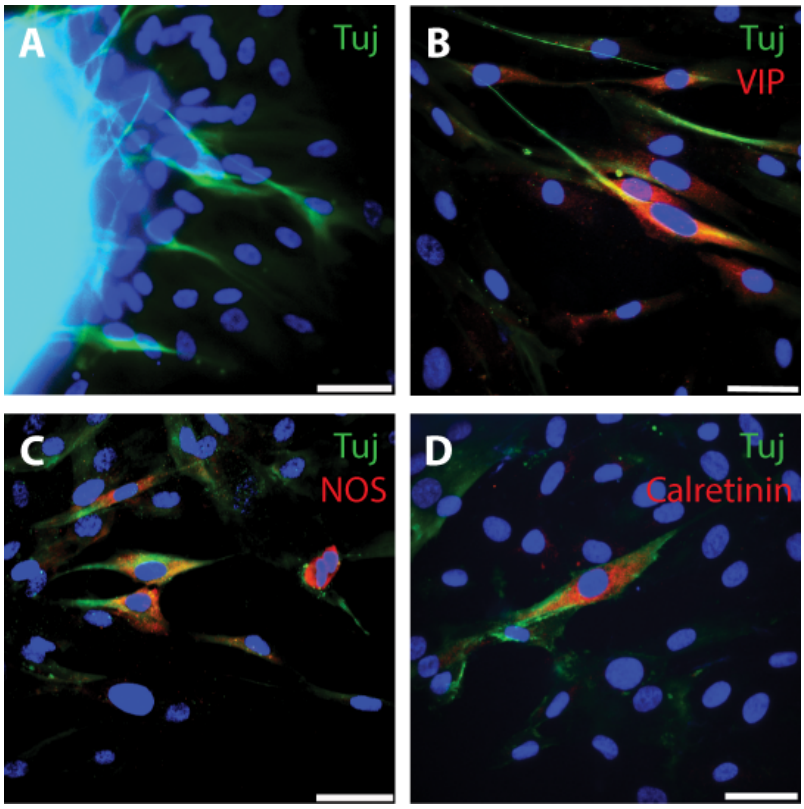


Figure 6.6. Neurons migrating away from human aganglionic-derived neurospheres express increasingly mature neuronal markers over time. (**A**) Cells migrating away from mature, tertiary human aganglionic derived neurospheres are labelled for the immature neuronal marker Tuj (green, β-tubulin III). By day 7 cells continued to express Tuj but cells were also identified expressing the mature ENS neuronal markers vasoactive intestinal peptide (VIP, **B**) and neuronal nitric oxide synthase (nNOS, **B**). Dual labelling of cells with Tuj and the ENS specific marker calretinin (red) was also noted **(D)**. Scale bars = 50µm.

6.2.6. Cell proliferation does not differ between HSCR aganglionic- or ganglionic-derived neurospheres

Having identified similarities in the expression profile of neurospheres derived from control patients and from ganglionic and aganglionic samples the next step was to look at cell behaviour. Following a 24h incubation period approximately 3% of cells within neurospheres were found to have incorporated EdU. No significant difference could be identified between neurospheres derived from control, ganglionic or aganglionic sources.

Figure 6.7. Rates of proliferation are not significantly different between human aganglionic- and ganglionic-derived neurospheres

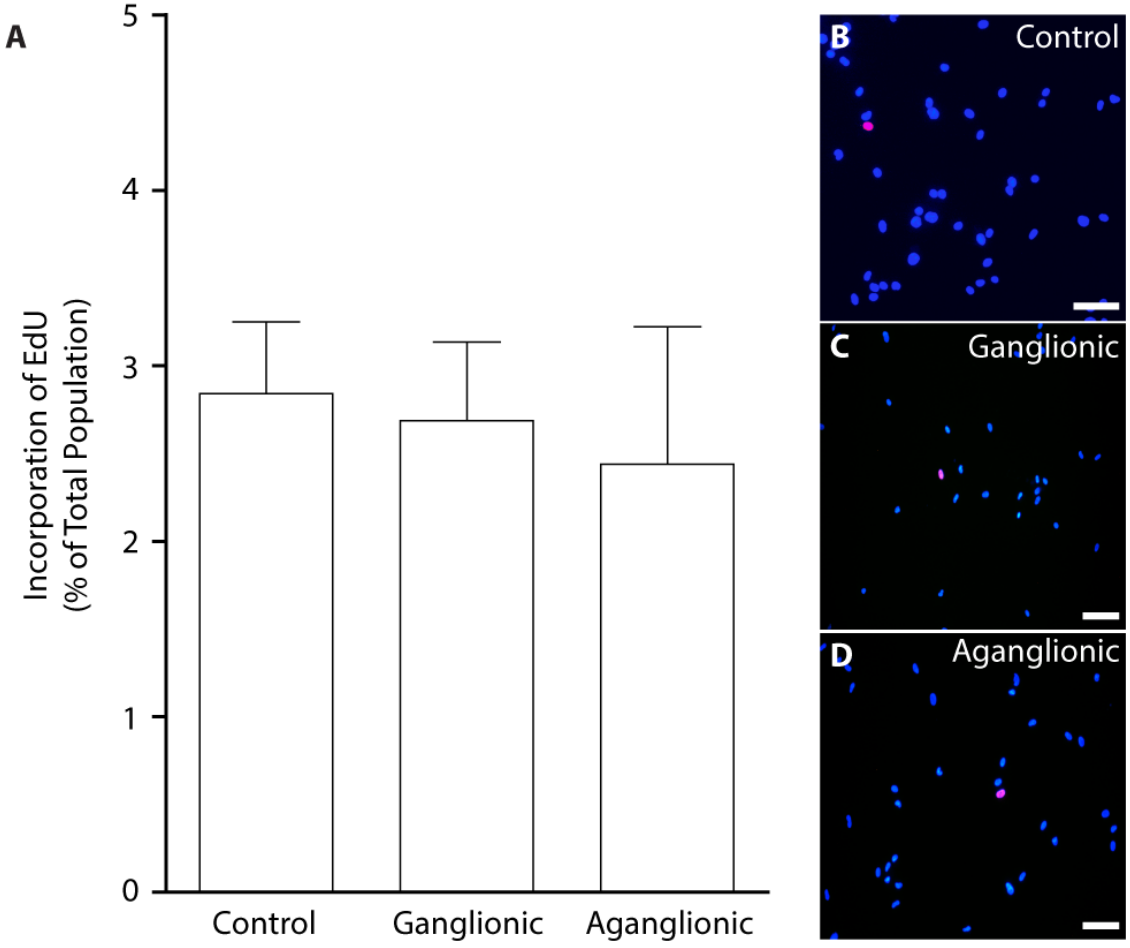


Figure 6.7. Primary neurospheres were isolated from 3-month old human controls, aganglionic colon and ganglionic HSCR colon. Primary neurospheres were cultured in the presence of EdU for 24h then immediately dissociated and spun down onto slides for fixation and analysis (**B-D**). No significant difference in incorporation of EdU was identified between human controls, aganglionic- or ganglionic-derived neurospheres (**A**, P>0.05). Error bars represent standard error of the mean (SEM) from 3 experiments. *Scale bars* = 50μm,

6.2.7. Transplanted HSCR aganglionic-derived neurospheres give rise to cells expressing immature neuronal markers

To test the functional potential of HSCR aganglionic-derived neural progenitors, neurospheres were transplanted into the ex-vivo aganglionic embryonic mouse model previously developed within the lab (Lindley, *et al* 2008). After 8 days in culture cells that had migrated away from the transplanted neurosphere and into the aganglionic embryonic colon were found to express the human specific marker HRNP. In addition, cells expressing the immature neuronal marker Tuj could also be identified in similar patterns to those found in ganglionic colonic explants (Figure 6.8). No neuronal cells were identified in control colonic explants that had not received neurosphere transplants.

Figure 6.8. Transplanted HSCR aganglionic-derived neurospheres give rise to cells expressing immature neuronal markers

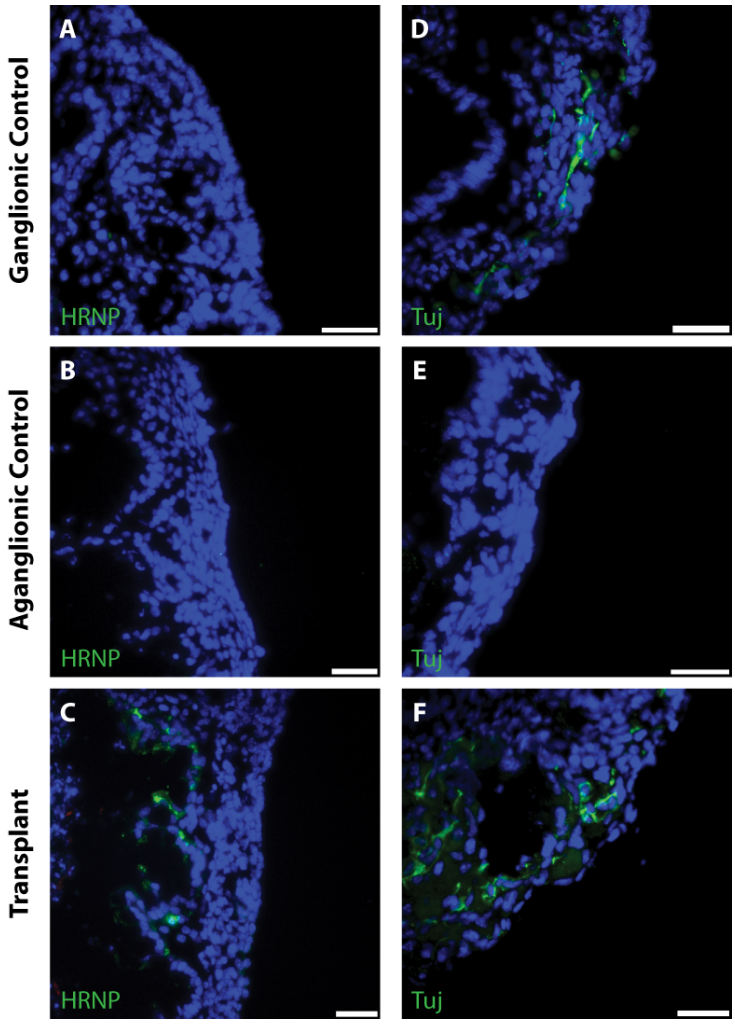


Figure 6.8. Cells from human HSCR aganglionic-derived neurospheres migrate into an embryonic aganglionic mouse colon. (**A to C**) Immunofluorescent labelling for human-specific ribonucleoprotein (green, HRNP) antibody after 8 days in culture were seen in the transplanted explants (**C**) but not in non-transplanted controls (**A,B**). (**E to G**) The immature neuronal marker Tuj was detected in both ganglionic controls (**D**) and the transplanted explants (**E**) but not in aganglionic controls (**F**). *Scale bars* = 50μm

6.2.8. Transplanted HSCR aganglionic-derived neurospheres can restore a normal pattern of contraction to an embryonic, aganglionic mouse model

Contractions were identified in all mouse colonic explants after 3-4days in culture, the pattern of which was determined using established motion capture techniques (Lindley*, et al* 2008). Contractions in control aganglionic explants were found to be short, irregular and would propagated only over short distances. In contrast contractions in both ganglionic controls (explants with the caecum intact allowing normal ENSPC migration) and aganglionic HSCR neurosphere transplants were regular, more sustained slower and could be seen to propagate over greater distances. Representative examples taken at day 8 are shown in Figure 6.9.

The frequency of contractions measured at the same time point was found to be significantly lower in human aganglionic-derived neurosphere transplanted explants compared to aganglionic controls. No significant difference was found between human aganglionic-derived transplants and ganglionic-derived controls (Figure 6.9). Taken together these results demonstrate that transplantation of human aganglionic-derived derived neurospheres can restore a normal pattern of contractility in the aganglionic model.

Figure 6.9. HSCR aganglionic-derived neurospheres can restore a normal pattern of contraction to an embryonic aganglionic mouse model

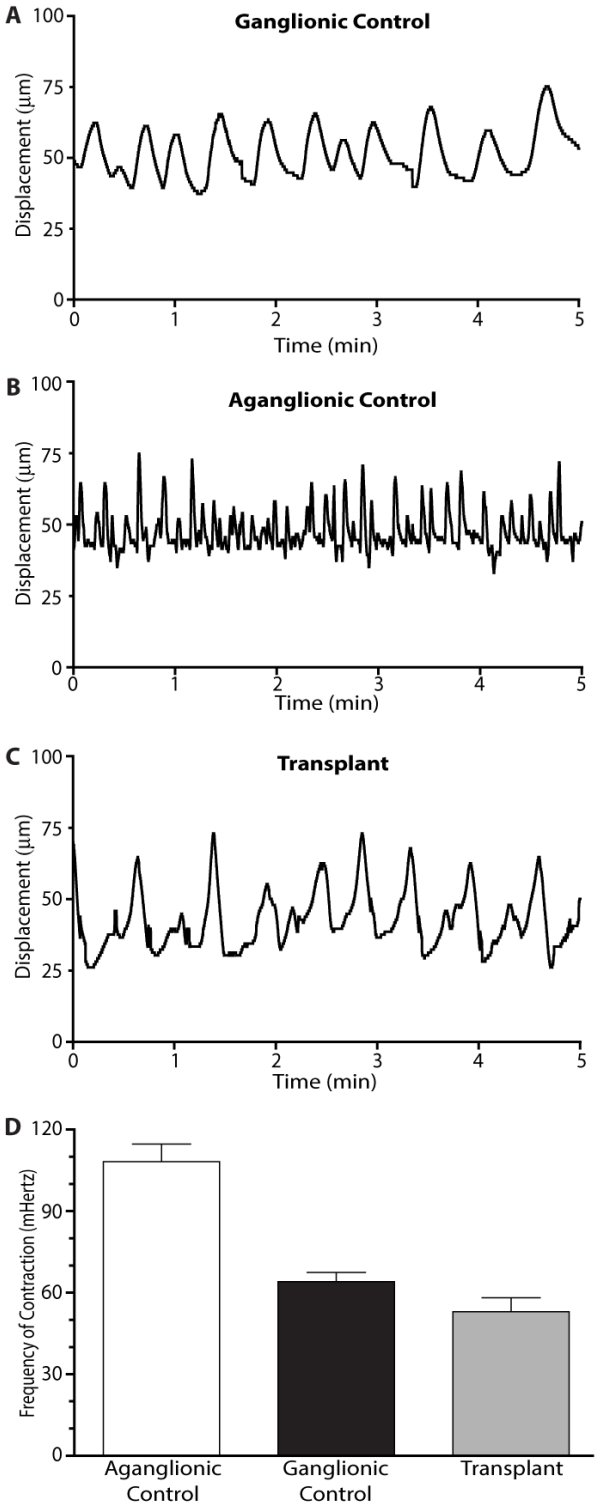


Figure 6.9. Cells from transplanted human aganglionic-derived neurospheres have the potential to restore a normal pattern of contraction to an embryonic, aganglionic mouse model. Explants were harvested at embryonic day 11.5 and analysed after 8 days in culture. The pattern of contraction was determined using image analysis software (Diamtrak, Prof Nield, Flinders University, Australia). (A) After 8 days in culture waves of contraction were identified in all distal ganglionic colonic explants. In aganglionic explants (no caecum / proximal colon) short (mean 3.1s), irregular contractions propagating over short distances were seen (B). Strikingly, at the same time-point in explants transplanted with aganglionic-derived neurospheres contractions appeared to be more co-ordinated with contractions found to be more regular, slower (mean 6.1s) and propagating over longer distances. These were similar to those seen in ganglionic controls (*A* and *C*). When the frequency of contraction was analysed no significant ($P>0.05$) difference could be found between ganglionic controls and human aganglionic-derived transplants (*D*). In contrast both of these conditions were associated with a significant ($p<0.001$) reduction in frequency when compared to aganglionic controls. n=5

6.3. Discussion

The most commonly accepted model for the development of HSCR is that there is a failure of vagal neural crest ENS progenitor cell migration (Yntema*, et al* 1954, Le Douarin*, et al* 1973, Young*, et al* 1998), resulting in the aganglionosis of the distal bowel. Surprisingly, the results in this chapter demonstrate that ENSPC-like cells can be isolated from aganglionic HSCR gut. These aganglionic progenitors express the same markers as their ganglionic counterparts and have the ability to differentiate into mature neuronal phenotypes. More importantly, when transplanted into an embryonic, aganglionic mouse model these cells can restore a normal pattern of contractility to the bowel. These observations have implications both for our understanding of the ENS developmental mechanisms involved in the pathogenesis of HSCR and also for development of novel therapies.

6.4.1. Aganglionic HSCR bowel contains neuronal progenitors

In this study we have been able to consistently isolate ENSPC-like cells from human smooth muscle biopsies taken from the aganglionic bowel of patients with HSCR, including those with long-segment disease. Importantly, the absence of enteric ganglia within these original aganglionic biopsies was confirmed both on H&E and by the absence of calretinin expression. Furthermore these findings have been corroborated by the clinical analysis of resected specimens by paediatric histopathologists, confirming the absence of components of the intrinsic nervous system in the regions where the biopsies were taken. When cultured under conditions promoting neuronal differentiation cells taken from aganglionic muscle biopsies form characteristic neurospheres that contain cells expressing markers of neural crest, neuronal and glial lineages.

These findings, coupled with the reproducibility of the findings in multiple patient samples makes the possibility of these findings being due to contamination from transitional or ganglionic bowel unlikely.

6.4.2. Cells from human aganglionic- and ganglionic-derived neurospheres differentiate to express neuronal markers characteristic of the ENS

We have shown that neurospheres derived from both the ganglionic and aganglionic HSCR biopsies contain progressively increasing proportions of cells expressing the neural crest marker P75 over time in culture, with a corresponding dramatic fall in the proportion of cells expressing smooth muscle markers after isolation. This suggests that cells within these neurospheres continue to develop or mature over time, ultimately resulting in neurospheres that predominantly contain cells expressing typical neural crest markers.

Perhaps most significantly of all the markers expressed by human aganglionic-derived neurospheres is the appearance of calretinin positivity. Calretinin is a calcium binding protein normally expressed by a subset of enteric ganglion cells within both the submucosal and myenteric plexi, and the absence of its expression is widely used a diagnostic test for HSCR (Palczewska*, et al.* 2005, Kapur*, et al.* 2009). The absence of calretinin expression in the initial aganglionic isolates was therefore expected, however its subsequent appearance in 1st generation neurospheres was surprising, albeit if it is slightly lower than the proportion found in ganglionic-derived neurospheres. Thus, the new neurons produced by aganglionic-derived neural progenitors have the defining characteristic used in clinical diagnosis of HSCR ganglionic and aganglionic gut. Furthermore, the nNOS and VIP neuronal phenotypes that developed from these cells are characteristic of ENS neurons expressing the typical neuronal markers seen in the ENS and calretinin.

6.4.3. ENSPC derived from aganglionic bowel can generate functional neurons

Previous work has shown that neurospheres derived from human ganglionic bowel are capable of restoring a normal pattern of contractility to an embryonic aganglionic mouse model (Lindley*, et al* 2008). Utilising this model, HSCR aganglionic-derived neurons have been found to demonstrate the same properties as ganglionic-derived controls. These results show that HSCR aganglionic-derived neurons have the ability to migrate into an embryonic aganglionic mouse model and more importantly, just like HSCR ganglionic-derived neurons, they can restore a normal pattern of contractility (Lindley*, et al* 2008). Thus, providing further support for a common source of neural progenitors in ganglionic and aganglionic bowel.

These observations bring in to question our current understanding of the precise aetiology of HSCR. It is a commonly held assumption that the lack of enteric neurons in the affected, distal aganglionic bowel is due to a failure of the enteric NC progenitors to migrate during development. This itself is supported by the studies of Simpson and Landman, *et al*. demonstrating that migration is largely driven by the 'population pressure' of NCCs at the rapidly proliferating wave front. Furthermore they suggest that the failure of colonisation seen in HSCR is therefore either due to inadequate proliferation at the migratory wave front or due to rapid bowel elongation (Simpson*, et al.* 2006, Landman*, et al* 2007, Simpson*, et al* 2007). However, the observations in this chapter suggest that there are progenitors present within the aganglionic bowel of children with HSCR that are capable of producing functioning components of the enteric nervous system. If these progenitors are present it begs the question as to why did they not differentiate during development? Along with the current theories regarding the cause of HSCR there may be a third factor - an inherent problem with the distal bowel preventing colonisation and differentiation of neural progenitors. Further study into the microenvironment of the aganglionic bowel in HSCR is warranted.

6.4.4. Progenitors in the aganglionic colon may arise from extrinsic nerve fibres

In culture the proportion of cells derived from human HSCR aganglionic gut expressing the more immature marker GFAP rose quickly. In contrast immediately after isolation the proportion of cells expressing the more mature glial and Schwann cell marker S100 (Bramanti*, et al.* 2010) was significantly higher than that of GFAP, and this ratio was quickly reversed in 1st generation neurospheres. The behaviour of glial markers in aganglionic HSCR cultures – in particular the relatively high number of cells expressing the S100 Schwann cell marker in cell isolates – prompted further us to further investigate the glia of aganglionic HSCR bowel.

Despite the absence of ENS neurons within the aganglionic regions of the bowel, one striking observation was the localisation of the neural crest marker P75 expression associated with extrinsic nerve fibres. These abnormally thickened fibres arise from the pelvic ganglia, which, in addition to the axons, contain p75-expressing cells of neural crest origin (Baumgarten*, et al* 1973, Kobayashi*, et al* 1994, Watanabe*, et al.* 1998). The high levels of P75 expression identified in the outer perineural cells of these nerve trunks are consistent with previous studies (Kobayashi*, et al* 1994). In addition lower levels of P75, are also expressed by the Schwann cells of the endoneurium characteristic of the Schwann cells of normal peripheral nerves (Kobayashi*, et al* 1994). Schwann cells have been well documented to show a high degree of plasticity in response injury or after experimental dissociation from peripheral nerves and culture. In particular, such treatments result in their proliferation, during or after which they have been shown to possess the capability of dedifferentiating to a multi-potent state and importantly have been shown to be capable of giving rise to new neurons in-vitro (Mirsky*, et al* 2008, Widera*, et al.* 2011).

The ability to isolate neural progenitors that possess properties similar to those derived from ganglionic colon is consistent with previous studies suggesting that ENSPC do not arise from within the enteric ganglia but instead from a separate extra-ganglionic niche (Liu*, et al*

2009). Although it has been often assumed that such progenitors would be derived from ENS ganglia (Kruger*, et al* 2002), a pulse-chase DNA labelling study provided evidence for the existence of a small population of proliferating extra-ganglionic cells that subsequently migrate into the ganglia of the postnatal gut in-vivo (Liu*, et al* 2009), although in the absence of suitable markers the identity of these cells and their progeny could not be established. Rather, these results are consistent with the progenitors originating from mature Schwann cells de-differentiating in response to the insult of dissociation. This would explain shift from the relatively higher expression of the mature glial/Schwann cell marker S100 in the fresh isolate to the high levels of GFAP expression within mature neurospheres. Interestingly, two recent studies looking at neurogenesis in the normal ganglionic postnatal ENS have shown that injury either by dissociation or application of benzalkonium chloride may stimulate neurogenesis in cells that express Sox10, GFAP and S100 – all of which may also be expressed by Schwann cells (Mirsky*, et al* 2008, Joseph*, et al* 2011, Laranjeira*, et al* 2011). This raises the possibility that Schwann cells may be the common ENS progenitor found in both ganglionic and aganglionic bowel.

These results support the hypothesis that the P75-positive cells of the extrinsic nerve trunks of aganglionic HSCR gut are the source of the progenitors identified arising from aganglionic biopsies. It should be noted that we have been unable to isolate such cells from the total aganglionic gut we were able to obtain from two very rare HSCR patients (Table 2.1). The total aganglionic gut has been reported to lack thickened extrinsic nerve trunks, and our inability to isolate ENSPC from it is consistent with a nerve trunk origin of the cells in short and long segment HSCR.

6.4. Conclusions

We have shown that neurospheres derived from both human ganglionic and aganglionic HSCR biopsies contain an increasing proportion of cells expressing the both the neural crest marker P75 and the immature glial marker GFAP with time in culture. Furthermore, cells within neurospheres derived from both ganglionic and aganglionic sources possess the capacity to differentiate into mature neuronal phenotypes expressing typical ENS specific markers and which can restore function to aganglionic embryonic gut ex-vivo. Clearly, the question remains as to whether these functional results can be translated to human postnatal bowel. However, this finding raises several interesting possibilities. The first is that it provides a source of progenitors for future autologous transplants in the treatment of HSCR. Secondly, if these progenitors are present throughout the aganglionic bowel it raises the question as to what is it in the local environment that prevents them from generating ENS neurons in-vivo and restoring a normal phenotype? Furthermore, it also opens up the possibility of developing future therapeutic modalities that stimulate neurogenesis in-vivo, which may reduce the need for surgery and ultimately improve outcomes for children with HSCR.

Chapter 7: Final conclusions

Human ENSPC, isolated from patients with HSCR, have previously been shown to be capable of restoring a normal pattern of contractility to an embryonic aganglionic mouse model (Lindley*, et al* 2008). This study, and more recent in-vivo transplantation studies in mice (Hotta*, et al* 2013) support the possibility of using these cells to restore function and thereby improve the current long-term morbidity encountered by patients with HSCR. The original aim of this thesis, building on previous work within the lab was to better understand the behaviour of ENPSC both in the environment of the neurosphere and in the microenvironment of human postnatal colonic smooth muscle, and subsequently to elicit the mechanisms controlling this behaviour. This knowledge would better enable us to predict how these cells might behave when transplanted into post-natal human bowel to both ensure the safety of any future cell-based therapy and allow us to maximise its effectiveness. Furthermore, this thesis reports the novel finding, arising from work pursuing the original aims, that ENSPC can be isolated from aganglionic bowel. This finding both questions our current understanding regarding the aetiology of HSCR and raises new therapeutic possibilities.

7.1. Embryonic ENSPC behaviour is not comparable to that of similar cells derived from the postnatal gut

The work within this thesis (chapter 3) supports previous studies which found little difference in the expression profiles of common ENS markers between embryonic mouse and postnatal human ENSPC in neurospheres (Almond*, et al* 2007). However, there was found to be a significant difference in the rate of proliferation seen in neurospheres derived from both sources. This difference appears to be due to the developmental maturation of the source tissue rather than the species, as not difference could be identified between mature postnatal mouse and human bowel. These results suggest that where human tissue is not available, or appropriate for experimental work that ENSPC derived from adult mouse (postnatal day 28) may be a more appropriate replacement than embryonic mouse ENSPC.

7.2. Notch signalling is involved in the regulation of both proliferation and differentiation of ENSPC

The work in this thesis demonstrates that ENSPC behave differently according to their position within neurospheres, e.g. proliferating cells are predominantly found at the periphery. These findings prompted further study into the mechanisms regulating this behaviour. Following work focusing on the control of normal ENS development in the mouse (Okamura*, et al* 2008, Ngan*, et al* 2011), we have now demonstrated that the Notch signalling pathway is implicated in the regulation of mature human ENSPC. Thus, Notch signalling is necessary to maintain a proliferative and undifferentiated state of ENSPC in tissue culture. These findings are of importance in terms of predicting and potentially regulating future transplanted ENSPC behaviour. When ENSPC are isolated from the colon there is a dramatic change in behaviour moving from an apparent quiescent state in-vivo to

that of rapid proliferation in culture. Before studies move towards human clinical trials we need to be able to know whether these cells will come back under of the host environment or whether they will progress towards unregulated proliferation and thereby develop the potential risk of tumour formation. Therefore, it is important to investigate the behaviour of transplanted human ENSPC in a controlled and safe environment.

7.3. Transplanted ENSPC behaviour can be investigated in a post-natal human aganglionic colonic smooth muscle model

Recent studies have reported that it is possible to transplant murine ENSPC into recipient mice and for these progenitors to migrate, interact with the host ENS and differentiate into electrically active neurons (Hotta*, et al* 2013). However, it remains important to determine how transplanted human ENSPC might behave in the microenvironment of post-natal human aganglionic smooth muscle. The work within this thesis (chapter 5) details the development of an ex-vivo model using human aganglionic smooth muscle seeded onto a decellularised mouse bowel scaffold. The ex-vivo model was found to contain cells that both expressed typical smooth markers and produced characteristic electrophysiological responses to administration of KCl and carbachol. Furthermore, the constructs could be reliably maintained in long-term culture and the behaviour of transplanted human ENSPC could be observed within them.

Although showing promise as a useful model in which to investigate both the behaviour and functional response of transplanted ENSPC further work is still required to validate the reliability of the model. The unexpected observation that, in in-vitro cultures, cells with an apparent neuronal phenotype were present within aganglionic samples meant that the optimisation of this model could not be completed within the timeframe of this thesis.

7.4. ENSPC can be isolated from HSCR aganglionic bowel

Human aganglionic bowel has been shown to contain progenitor cells that are capable of differentiating into functional mature ENS-specific neurons (chapter 6). These novel findings question our current understanding of the aetiology behind HSCR such as the distal bowel is aganglionic because of a failure of craniocaudal migration of NCC progenitors during development. If this is true why are progenitors present in the aganglionic bowel that are capable of differentiating into ENS neurons? Why have these progenitors failed to differentiate in-vivo? These results suggest that there may be an intrinsic defect in the aganglionic bowel that is preventing existing progenitors developing into function enteric neurons.

The results also raise the possibility of utilising these progenitors for therapeutic benefit. Progenitors isolated from the aganglionic bowel could be used as part of an autologous transplantation therapy into the internal anal sphincter after surgery. The aim is analogous to that of delivering dopaminergic neurons in Parkinson's disease (Fan*, et al.* 2013) delivering neurons and thereby the missing neurotransmitter to this aganglionic region that cannot be resected. But perhaps more interestingly, it raises the potential to manipulate the microenvironment in-vivo to stimulate progenitor cell differentiation into mature ENS neurons in-situ and in-vivo.

7.5. Final conclusion

The work in this thesis furthers our understanding of the control and behaviour of human ENPSC, specifically within the ex-vivo environment of the neurosphere. But perhaps most significantly, this thesis describes the discovery of previously unknown ENS progenitors in the aganglionic bowel of children with HSCR. This raises questions about the aetiology of HSCR, the source of ENSPC in the ganglionic bowel and the potential to use these cells in future cell-based therapies.

7.6. Future work

The findings within this thesis remain consistent with a potential therapeutic role for ENPSC in treating conditions such as HSCR. However, significant work still remains to be done to ensure the safety and optimise the effectiveness of any future cell-based transplantation therapy.

7.6.1. Role of Notch signalling post-transplantation

The work in chapter 4 details the significant role of Notch signalling in the control of ENSPC behaviour in the neurosphere. This chapter also demonstrates the ability to determine its activation state using the intracellular expression of NICD (Figures 4.12/13). Using this technique and others such as expression of downstream proteins, e.g. HES (Theocharatos*, et al* 2013), or live imaging with a luciferase based promoter (Ilagan*, et al* 2011) would allow assessment of the state of Notch signalling in ENSPC both pre and post transplantation. Using the ex-vivo human colonic smooth muscle model described in chapter 5, it may be possible to determine whether Notch signalling is switched off after transplantation thereby limiting proliferation and driving cells towards a neuronal lineage. Furthermore, if the rate of proliferation remains high in transplanted ENSPC, it may be possible to manipulate the Notch pathway to inhibit this proliferative behaviour and minimise the risk of tumour formation.

7.6.2. Development of the human colonic smooth muscle model

Due to the unexpected findings detailed in chapters 5 and 6 it was not possible to fully optimise the human colonic smooth muscle model (HCSMM). The provisional data contained in chapter 5 does suggest that there is a role for this model in bridging the gap between animal model studies and in-vivo human clinical trials. Further work is required to

optimise the seeding density and time in culture to produce a model that generates the maximum resting force possible while remaining stable in culture. This will allow the functional effect of ENPSC transplantation to be more easily identified. It is also important to determine the reproducibility of this model, i.e. does a model seeded with the same density of cells as another possess the same electrophysiological properties as another. Determining this degree of variability is required again so that we can be certain that a difference in physiological response is due to the transplanted cells and not to intermodal variability. Once these factors are optimised it should then be possible to transplant ENSPC, track their integration and determine their functional potential in a safe, reproducible environment that is closer to that of the human colon.

7.6.3. The optimum method of ENSPC transplantation

ENSPC have previously been transplanted in-vivo either directly into the peritoneum or by direct placement into the muscular wall of the colon (Micci*, et al* 2005, Tsai*, et al* 2010, Hotta*, et al* 2013). The most promising results are seen with the direct placement of ENSPC, but alternative methods such as microinjection directly into the internal anal sphincter under endoscopic or ultrasound control, need to be evaluated for their effectiveness prior to clinical trials.

7.6.4. Origin of ENSPC in the aganglionic bowel

The results in chapter 6 suggest that the progenitors identified in the aganglionic bowel may be related to the abnormally thickened nerve trunks seen in these regions, however these results require further confirmation. Given that expression of the neural crest marker P75 is limited to these fibres it should be possible to use flow cytometry assisted cell sorting to isolate P75+ve cells from the initial cell isolate produced when human colonic biopsies are processed. If these cells can then be shown to go on to differentiate into the same

mature neurons as seen in chapter 6 it would confirm the relationship of these progenitors to the abnormal nerve fibres. However, although the relationship would be confirmed it would still not explain their origin.

7.6.5. Stimulating ENSPC differentiation in aganglionic bowel

Groups have already started to investigate the possibility of manipulating the local microenvironment to stimulate proliferation and neuronal migration into aganglionic areas from the neighbouring ganglionic bowel (Matsuyoshi*, et al* 2010, Laranjeira*, et al* 2011). However, now having identified the presence of progenitors in the aganglionic HSCR bowel the question remains why did these cells not differentiate into mature neurons during development and therefore, can they now be stimulated to differentiate in-vivo? Experiments are needed in mouse models of HSCR, firstly to confirm the presence of ENS progenitors in the aganglionic bowel of these mice and subsequently to determine whether the addition of agents known to affect ENSPC behaviour such a $5HT_4$, GDNF, ET3 can influence neuronal differentiation in aganglionic bowel.

7.7. Publications arising from this thesis

Publications arising from this thesis are included in the appendix at the end of the thesis. A manuscript reporting the results of chapter 6 is currently being prepared.

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Appendix: Papers arising

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PLOS ONE

Regulation of Progenitor Cell Proliferation and Neuronal Differentiation in Enteric Nervous System Neurospheres

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Abstract

Enteric nervous system (ENS) progenitor cells isolated from mouse and human bowel can be cultured *in vitro* as neurospheres which are aggregates of the proliferating progenitor cells, together with neurons and glial cells derived from them. To investigate the factors regulating progenitor cell proliferation and differentiation, we first characterised cell proliferation in mouse ENS neurospheres by pulse chase experiments using thymidine analogs. We demonstrate rapid and continuous cell proliferation near the neurosphere periphery, after which postmitotic cells move away from the periphery to become distributed throughout the neurosphere. While many proliferating cells expressed glial markers, expression of the neuronal markers β-tubulin III (Tuj1) and nitric oxide synthase was detected in increasing numbers of post-mitotic cells after a delay of several days. Treatment of both mouse and human neurospheres with the γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) reduced expression of the transcription factors Hes1 and Hes5, demonstrating inhibition of Notch signaling. DAPT treatment also inhibited progenitor cell proliferation and increased the numbers of differentiating neurons expressing Tuj1 and nitric oxide synthase. To confirm that the cellular effects of DAPT treatment were due to inhibition of Notch signaling, siRNA knockdown of RBPjκ, a key component of the canonical Notch signaling pathway, was demonstrated both to reduce proliferation and to increase neuronal differentiation in neurosphere cells. These observations indicate that Notch signaling promotes progenitor cell proliferation and inhibits neuronal differentiation in ENS neurospheres.

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Introduction

During vertebrate embryonic development, enteric nervous system (ENS) progenitor cells arising primarily from the vagal region of the neural crest migrate rostrocaudally along the gut, proliferating and differentiating to form the ganglia of the ENS [1,2,3]. Failure of this migration in humans results in Hirschsprung's disease (HSCR), characterised by intestinal aganglionosis, which typically extends to a variable extent rostrally to include the internal anal sphincter, rectum and distal colon [4]. The absence of the ENS in the distal bowel causes a smooth muscle constriction that in turn gives rise to the megacolon seen in neonatal HSCR patients. Current treatment involves surgical resection of the aganglionic gut, but a high proportion of patients continue to experience postoperative morbidity [5], which may result from the small region of residual aganglionic distal bowel that always remains after surgery [4]. In recent years several groups have begun to assess the feasibility of using ENS progenitor cells for future use to provide a source of neurons to improve the function of this residual aganglionic gut [6].

We and others have isolated ENS progenitor cells from human and mouse gut and begun to characterise their properties both *in vitro* and after transplantation [7,8,9,10]. Typically, the cells are grown in culture as aggregates known as neurospheres, by analogy with the neurosphere cultures previously described for stem cells derived from the central nervous system (CNS) [11,12]. Both CNS and ENS neurospheres contain multipotent self-renewing neural progenitor cells and their neuronal and glial progeny [7,11]. Significantly, ENS neurosphere transplantation into *ex vivo* explants of aganglionic embryonic gut restored a normal pattern of contractility [13].

It is essential to understand the mechanisms controlling progenitor cell proliferation, self-renewal and differentiation in neurospheres before the cells can be used safely for transplantation therapy, as continuing proliferation after transplantation could result in tumor formation. Clearly the niche provided by neurospheres in culture differs from that of ENS ganglia *in vivo*, and this difference is likely to be the reason for the proliferative behavior of the cells in neurospheres. It has been well documented that the proliferation and differentiation of a variety of neural

progenitor cells can be regulated by the Notch signaling pathway [14,15,16]. While there is some evidence consistent with the need for Notch signaling during ENS development [17,18], it remains to be established if Notch signaling can regulate the proliferation and differentiation of ENS progenitor cells.

As a prerequisite for future analysis of neurosphere cell behavior in vivo after transplantation, the work reported here characterizes cell proliferation and neuronal differentiation in ENS-derived neurospheres, and then investigates mechanisms controlling that behavior *in vitro*. We show that cells proliferate rapidly at or near the periphery of the neurosphere, after which postmitotic cells migrate throughout the neurosphere. While few cells expressing neuronal markers were found to be actively proliferating, expression of the markers increased several days after leaving the cell cycle. We furthermore demonstrate using chemical and siRNA inhibition that Notch signaling is necessary both for the maintenance of cell proliferation and suppression of neuronal differentiation in ENS neurospheres.

Materials and Methods

Ethics Statement

In accordance with the United Kingdom Animal (Scientific Procedures) Act of 1986, this study did not require a Home Office project license because no regulated procedures were carried out. Mice were humanely killed at a designated establishment by CO_2 asphyxiation, which is an appropriate method under Schedule 1 of the Act. Ethical approval for the isolation of human ENS progenitor cells was given by the North West 3 Research Ethics Committee (Ref: 10/H1002/77). Written parental consent was obtained before samples were taken.

Mouse ENS Neurosphere Preparation

Time mated CD-1 mice (Charles River Laboratories, UK) were sacrificed 11.5 days post-coitum by inhalation of increasing concentrations of carbon dioxide. The preparation of ENS neurospheres has been described in detail previously [7,13]. Briefly, dissected ceca were incubated with 0.05% (w/v) trypsin (Sigma Aldrich, UK) in Dulbecco's phosphate buffered saline (PBS, Invitrogen, Life Technologies, UK) for 15 min at 37°C. After mechanical dissociation, $2–3\times10^6$ cells were transferred to 60 mm non-adherent culture dishes (Sterilin, ThermoFisher Scientific, UK) in 4 ml Dulbecco's modified Eagle medium (1 mg/ml glucose), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Life Technologies, UK), 2 mM L-glutamine (Invitrogen, Life Technologies, UK), 2% v/v chick embryo extract (Sera Laboratories Int., UK), 1% (v/v) N1-supplement (Sigma-Aldrich, UK), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, UK), 20 ng/ml, EGF (Sigma-Aldrich, UK) and 20 ng/ml FGF2 (Autogen Bioclear, UK). The culture medium was replaced every 96 h, and after 2 weeks the suspended neurospheres had reached diameters of about 100 µm.

Human ENS Neurosphere Preparation

Human neurospheres were generated from the ganglionic colon of neonates undergoing elective abdominal surgery as previously described [7,13]. Briefly, after removing the mucosa and submucosa from 1 cm^2 full thickness gut samples, the muscle layers were mechanically disrupted into 1–2 mm^2 pieces. This was followed by 1 h incubation with 0.5% (w/v) collagenase and 0.5% (w/v) dispase (Gibco, Life Technologies, UK) in PBS at 37°C before trituration. The incubation step was repeated 2–4 times with fresh enzyme solutions until a single cell suspension was obtained. The cell suspension was then cultured under the same conditions as used to generate mouse neurospheres. The human neurospheres were used when they had reached either the secondary or tertiary generation, both of which have been previously characterized [19].

Formation of Chimeric Neurospheres

A single-cell suspension was prepared by dissociation of 2–3 week old mouse neurospheres by trypsinization (0.05% w/v trypsin in PBS for 10 min) and trituration. Constitutive expression of enhanced green fluorescent protein (eGFP) under control of the spleen focus-forming viral (SFFV) promoter was by Lentiviral transduction. After the cells had begun to express eGFP (2- days), chimeric neurospheres were produced by centrifuging 5×10^3 labeled cells at 150 g onto aliquots of unlabeled neurospheres taken from the same batch as that used to obtain cells for viral transduction. The chimeric neurospheres were maintained in suspension culture for a further 96 h before fixation.

Immunostaining

Neurospheres were transferred into Shandon Cryomatrix (Thermo Fischer Scientific, UK) and stored at −80°C until 8 µm serial frozen sections were prepared by cryostat. For single cell analysis, the neurospheres were dissociated by trypsinization and trituration. 5×10^3 aliquots of cells were allowed to attach to Permanox 8-chamber culture slides (Sigma Aldrich, UK) for 3 h before fixation.

Neurosphere sections and single cells were fixed with 4% (w/v) paraformaldehyde (PFA) followed by permeabilization with 0.25% (w/v) Triton X-100 in PBS (Sigma-Aldrich, UK). After rinsing and blocking with 2% (w/v) bovine serum albumin (Sigma Aldrich, UK) in PBS, primary antibodies were applied at the following dilutions in the blocking buffer: rabbit anti-p75 (Abcam, UK) 1:500; rabbit anti-GFAP (DAKO, UK) 1:1000; mouse anti-GFAP (Sigma-Aldrich, UK) 1:1000; goat anti-Sox10 (Santa Cruz, USA) 1:100; mouse anti-Tuj1 (Abcam, UK) 1:500; rabbit anti-S100 (Abcam, UK) 1:800; rabbit anti-NOS (Abcam, UK) 1:800. Isotype controls were performed with the same concentrations of non-immune antibodies. After incubation overnight at 4°C, samples were rinsed, followed by 2 h incubation with the appropriate secondary antibodies diluted 1:1000 in blocking buffer (all from Invitrogen, Life Technologies, UK). All primary antibodies react with mouse and human antigens.

Assessment of immunoreactivity was made using standard fluorescence or confocal microscopy where specifically stated. Counting of immunopositive and EdU-positive cells was undertaken in >5 random optical fields across each chamber using a standard fluorescence microscope and 40x oil objective.

BrdU and EdU Incorporation

Two to 3 week old mouse neurospheres were incubated in culture medium containing 10 µM bromodeoxyuridine (BrdU) for the times shown. For BrdU staining, frozen fixed neurosphere sections were treated with 4 M HCl for 15 min and rinsed with distilled water prior to permeabilization and immunostaining for nuclear BrdU (DAKO, UK). For cells dissociated from neurospheres, proliferation was assessed by incubation with 10 µM ethynyldeoxyuridine (EdU) for 1 h immediately before dissociation to single cells (this procedure does not use HCl treatment and so helps preserve the morphology of cells). Dissociated cells were allowed to attach briefly to Permanox culture slides before processing according to the manufacturer's instructions to visualize nuclear EdU by the binding of the azide group of the Click-it® Alexa594 fluorophore to the alkyne group of EdU (Click-it EdU Imaging Kit, Invitrogen, Life Technologies, UK).

Inhibition of Notch Signaling

Preliminary experiments investigating the effects of Notch inhibition used the γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Sigma Aldrich, UK), dissolved in dimethyl sulfoxide (DMSO). An equal volume (5 µl) of DMSO was applied to control dishes. For determination of proliferation and expression of neuronal and glial markers, cells were dissociated from the DAPT-treated neurospheres and controls by trypsinization and trituration, after which they were allowed to attach to Permanox chamber slides in culture medium before fixation with 4% (w/v) PFA.

siRNA knockdown of RBPjκ was performed with human neurosphere cells attached to Permanox chamber slides using the following oligomers (Qiagen, UK): HsRBPJ_1 (TAGGGAAGCTATGCFAAATTA); HsRBPJ-2 (GTGGCTGGAATACAAGTTGAA); HsRBPJ_3 (CACGGTATTATAGTACACCTT). The control oligomers used were the Qiagen All Stars Human Cell Death Control® and All Stars Negative Control®. Transfection was performed according to the manufacturer's instructions using a HiPerFect® transfection kit (Qiagen, UK). The transfection reagent was used at a concentration of 3 µl/ml with a final oligomer concentration of 10 nM. Determination of Tuj1 expression and EdU incorporation was performed after 96 h as described above.

qPCR

RNA was extracted using the Trizol® Reagent (Invitrogen, Life Technologies, UK) according to the manufacturer's instructions, using 20 µg/µl glycogen (Invitrogen, Life Technologies, UK) as carrier. Extracted RNA was treated with 1 U/µl RQ1 DNase (Promega, UK) before cDNA synthesis with Superscript® III reverse transcriptase (Promega, UK). The qPCR reaction using a Corbett Rotor-Gene RG-3000 thermal cycler (Qiagen, UK), was with KAPA-SYBR® hot start master mix (KAPA BIOSYSTEMS, UK).

The primers used to determine levels of mouse Hes1 and Hes5 mRNA were: Hes1: GCACAGAAAGTCATCAAAGCC forwards, TTGATCTGGGTCATGCAGTTG reverse; Hes5: AGTCCCAAGGAGAAAAACCGA forwards, GCTGTGTTTCAGGTAGCTGAC reverse, β-actin: CGTTGACATCCGTAAAGACC forwards, CAGGAGGAGCAATGATCTTGA reverse. For human RBPjκ mRNA, a QuantiTect® kit primer assay for RBPjκ (QT01680049, Qiagen, UK) was used according to the manufacturer's protocol. PCR products were analysed by agarose gel electrophoresis, melting curves and sequencing. Expression of the target genes relative to β-actin mRNA was determined using the comparative C_t method [20].

Data Analysis

Statistical analysis was performed using GraphPad Prism Version 5 (GraphPad Software, USA). Significance was determined by Student t-test or ANOVA as indicated in figure legends. Results are expressed as mean ± SEM.

Results

Analysis of Neurosphere Cell Proliferation and Migration

Two to 3 week old primary mouse neurospheres were cultured for up to 96 h in medium containing 10 µM BrdU. Photomicrographs were recorded of sections cut through the centre of each neurosphere, which were identified as the serial sections with the greatest diameter. After 6 h incubation, BrdU incorporation was restricted to a few cells at or near the periphery of the neurosphere (Fig. 1A). In contrast, by 96 h many of the nuclei had incorporated

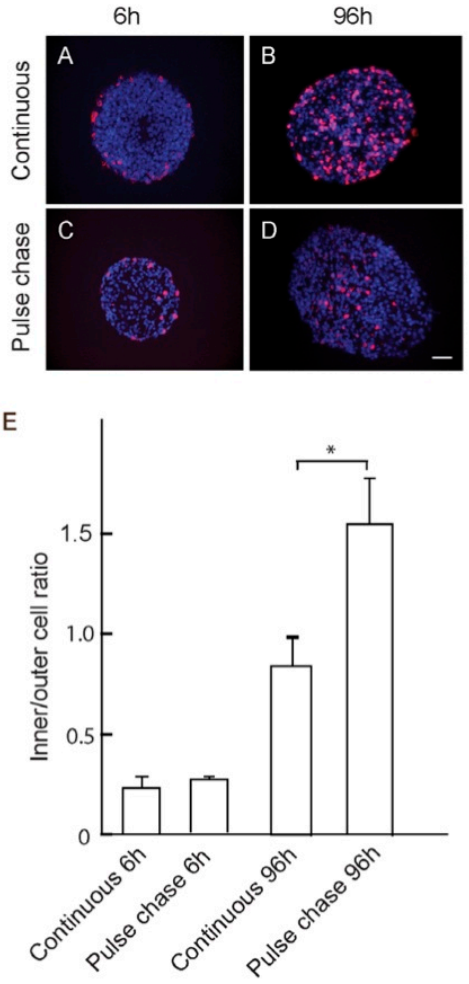

Figure 1. Cell proliferation in mouse neurospheres after continuous labeling and pulse-chase with BrdU. A, B: Continuous labelling with 10 µM BrdU after 6 h (A) and 96 h (B) culture. C, D: Labelling after 1 h pulse of 10 µM BrdU, neurospheres immediately after the pulse (C), and 96 h after chase in absence of BrdU (D). Images show immunostaining for BrdU of sections taken through the equatorial region of the neurospheres, counterstained with DAPI. E: Ratio of BrdU stained nuclei in the inner 50%/outer 50% of the area of the sections. Values are means±SEM (n>5) from 2 independent experiments. *p<0.01. Scale bar = 25 µm.
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BrdU (Fig. 1B). Moreover, the labeled cells were distributed uniformally throughout the neurosphere (Fig. 1E).

Pulse-chase analysis confirmed initial incorporation of BrdU into cells at or near the neurosphere periphery immediately after a 1 h pulse (Fig. 1C). However, after a 96 h chase, very few weakly labeled cells could be detected at the periphery while strongly labeled nuclei were now distributed toward the centre of the neurosphere (Fig. 1D, E). Images produced during the chase in the absence of BrdU showed that the level of labelling of nuclei at the periphery of the neurosphere gradually decreased with time to become virtually undetectable by 96 h, while strongly labeled nuclei were located nearer the centre of the neurosphere (Fig. 1D

and Fig. S1). These observations indicate that cells remaining at or near the neurosphere periphery continue to proliferate while postmitotic cells move from the periphery to become distributed throughout the neurosphere.

To demonstrate directly that cells move within the neurospheres, we constructed chimeric neurospheres in which about half the cells were labeled with the constitutively expressed eGFP (Fig. 2A). After 96 h, the initially sharp boundary between GFP -positive and –negative cells became indistinct in living whole mount preparations (Fig. 2B). Analysis of fixed frozen sections at this time point showed that eGFP -positive cells had migrated into the unlabeled neurosphere halves (Fig. 2C). Furthermore, cell proliferation was again restricted to the periphery of both halves of the chimeric neurosphere while being absent from the interface between eGFP –positive and –negative cells (Fig. 2C). These observations demonstrate directly that there is indeed cell movement within the neurosphere.

Relationship between Cell Proliferation and Differentiation

Confocal immunofluorescence of equatorial neurosphere sections showed that the majority the cells expressed p75, consistent with their neural crest origin (Fig. 3A), whereas cells expressing the ENS progenitor and glial marker Sox10 were distributed sparsely throughout the neurosphere (Fig. 3C), as were the glial markers GFAP and S100 (Fig. 3B and D). In contrast, immunoreactivities of the neuronal markers, neuronal Class III β-Tubulin (Tuj1) and nitric oxide synthase (NOS) were mainly located near the neurosphere periphery and only a few sporadic cells or fibers were seen throughout the neurosphere (Fig. 3E and F). Higher power confocal microscopy showed that the subcellular localization of NOS and Tuj1 immunoreactivity was difficult to define, as these neuronal markers appeared to be in both cell bodies and fibers (Fig. 3G and H).

Because it was difficult to quantitate numbers of Tuj1- and NOS-expressing cells in the neurospheres (see Fig. 3), we dissociated the neurospheres to single cells which were then allowed to attach to adhesive substrates in order to double label and count cells for marker expression and proliferation demonstrated by incorporation of the thymidine analog EdU (Fig. S2). Very few cells expressing the neuronal markers Tuj1 (<8%) or NOS (<2%) had incorporated EdU immediately after the pulse (Fig. 4). Significantly, 96 h after EdU labeling, the proportion of labeled cells expressing Tuj1 and NOS neuronal markers had increased about 5-fold (Fig. 4), indicating that postmitotic progenitor cells differentiate to acquire a neuronal phenotype with a delay of several days. Although increased numbers of labeled cells expressing the glial markers 96 h after EdU labeling were also found, this increase was less than that found for the neuronal cells, due in part to the relatively high proportion of glial cells that were expressing these markers during or immediately after the labeling (Fig. 4).

Role of Notch Signaling in Neurosphere Cell Proliferation and Differentiation

Initial experiments implicating Notch signaling in the proliferation and differentiation of neurosphere cells utilized the chemical inhibitor of γ-secretase DAPT, which blocks Notch signaling by inhibiting the cleavage of the Notch intracellular signaling peptide NICD [21]. Incubation of mouse neurospheres for 96 h with DAPT reduced mRNA levels of the transcription factors Hes1 and Hes5 which are downstream targets of NICD in the canonical Notch signaling pathway (Fig. 5A and Fig. S3). The level of Hes1 was reduced by 50%, and Hes5 decreased by almost 90% relative to controls, indicating an effective block of Notch signaling. Significantly, γ-secretase inhibition reduced neurosphere cell proliferation to 55% of controls (Fig. 5B), while the numbers of Tuj1-positive and NOS-positive neuronal cells increased. In contrast, the numbers of cells expressing the glial cell marker S100 did not change significantly (Fig. 5B). Similarly, DAPT treatment reduced the proliferation of cells dissociated from human neurospheres while increasing the numbers of Tuj1-positive cells present (Fig. 6A and 6C).

Although these results are consistent with a role for Notch signaling in the regulation of ENS progenitor cell proliferation and differentiation in neurospheres, they do not prove it because inhibition of γ-secretase may affect other signaling pathways with which it is involved [21]. In order to demonstrate that Notch signaling does indeed regulate neurosphere cell proliferation and differentiation, we investigated the effects of siRNA knock-down of RBPjκ (a key component of Notch signaling [22]). The effectiveness of the knockdown was determined by a reduction of mRNA levels of RBPjκ after siRNA treatment using the HsRBPJ_3 oligomer to about one third of control levels in human neurosphere cells (Fig S4). Similar to the effects of DAPT treatment of these cells, siRNA knockdown of RBPjκ with the HsRBPJ_3 siRNA resulted in a three-fold reduction of proliferation (Fig. 6B) and greater than five-fold increase in expression of the early neuronal marker Tuj1 (Fig. 6D). Similar decreases were found with the other two siRNAs directed against RBPjκ, HsRBPJ_1 and HsRBPJ_2 (data not shown). Taken together, these results show that Notch signaling is necessary for progenitor cell proliferation and inhibition of neuronal differentiation in ENS neurospheres.

Discussion

Although neurosphere culture offers a potential method for the amplification of ENS progenitor cells for future transplantation therapies in HSCR [6,8,13], there have been no studies into why the cells proliferate rapidly in neurospheres, nor why some of them cease proliferation and differentiate into postmitotic neural cells. This knowledge will be necessary for future work to ensure that ENS cells do not proliferate uncontrollably after transplantation, and also to optimise therapy by promoting the differentiation of the most functionally effective neuronal subtypes. Our investigations here demonstrate that rapid cell proliferation occurs at or near the periphery of ENS neurospheres, after which postmitotic cells move throughout the neurosphere. Neuronal differentiation occurs after the progenitor cells cease proliferation, and both proliferation and neuronal differentiation of ENS neurosphere cells are regulated by Notch signaling.

ENS progenitor cells have previously been isolated and cultured by a variety of methods that differ in the sources of cells, methods of isolation and tissue culture techniques used to grow them [7,8,9,10]. These variations may well be responsible for differences in the properties of the cells reported. For example, it is known that ENS progenitor cells isolated from developing gut have more restricted differentiation potential as they mature [23]. Furthermore it has been shown recently that neurosphere-like bodies isolated from submucosal and myenteric regions of the bowel contain distinct subpopulations of cells that differ in their functions and phenotypes [9]. Thus, neurosphere-like bodies derived from postnatal human submucosal tissue have been demonstrated to comprise a majority of mesenchymal non-neural cells and their progenitors, necessitating cell purification techniques to enrich the small subpopulation of ENS cells present [9,24]. In contrast, neurospheres derived from embryonic mouse gut and from the myenteric region of postnatal human bowel give rise to neuro-

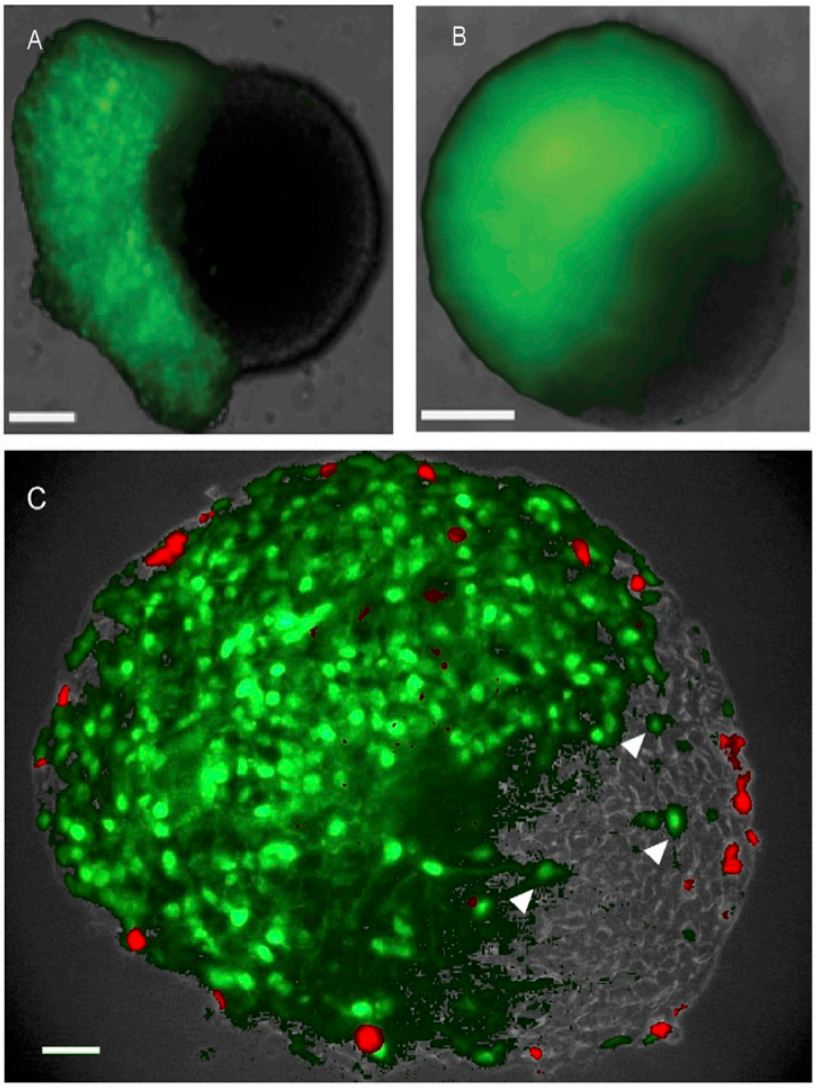

Figure 2. Cell migration and proliferation in chimeric mouse neurospheres. eGFP expressing neurosphere cells were centrifuged onto unlabeled intact neurospheres to produce chimeric neurospheres. Wholemount fluorescence images of living neurospheres taken (A) after 24 h and (B) after 96 h culture, showing green eGFP fluorescence: note that after 96 h the boundary between labeled and unlabeled cells has become diffuse. C, 8 μm equatorial section of typical chimeric neurosphere after 96 h culture, at the end of which the neurospheres had been incubated with 10 μM BrdU for 1 hour before fixation. Note that eGFP cells have migrated into the unlabeled half of the chimeric neurosphere (C, arrow heads), and that BrdU labelling (red) is restricted to the periphery of the chimeric neurosphere. Scale bars = 100 μm.
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spheres in which the majority of cells express the p75 marker (see Fig. 4A), consistent with their neural crest origin [7,13,19].

We previously demonstrated the presence of multipotent progenitor cells in neurospheres derived both from embryonic mouse gut and from postnatal human bowel [7,19]. While this work showed that the progenitor cells retain similar differentiative and proliferative properties over a period of months in culture [7,19], ENS neurosphere cell proliferation and its relationship to differentiation has not been investigated in any detail. We demonstrate here by pulse-chase experiments with thymidine analogs that a brief exposure to BrdU results in a rapid initial labelling of cells at or near the neurosphere periphery. Although the peripheral location of BrdU incorporation may be due to its inability to penetrate completely into the neurosphere during this brief one hour labelling period, it is significant that the level of labelling of the peripheral cells gradually declines with time after the pulse of BrdU, while heavily labeled cells are found distributed throughout the neurosphere (see Fig. 1). Given our demonstration that there is considerable mixing of cells in chimeric neurospheres (see Fig. 2), then the simplest interpretation of these observations is that the progeny of cells that initially divided at the neurosphere periphery slowed or stopped proliferation as they migrated

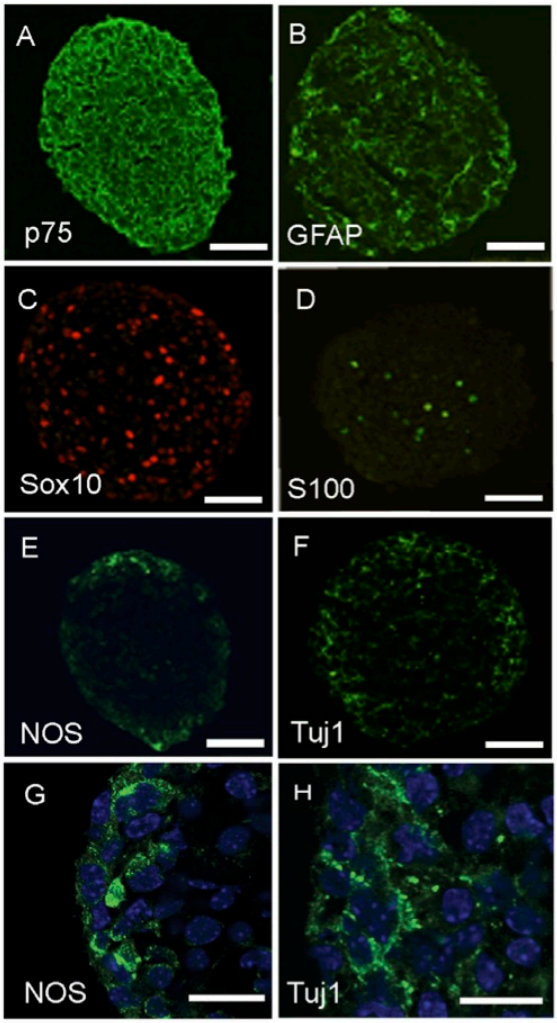

Figure 3. Expression of progenitor, glial and neuronal markers by mouse neurosphere cells. Primary neurospheres were fixed after 15 days culture and 8 μm equatorial sections produced. Confocal immunofluorescence images are shown. G and H are higher magnifications of the peripheral immunofluorescence for NOS and Tuj1 shown in E and F, respectively. Note that NOS and TuJ1 immunofluorescence is located both in cell bodies and fibers (G,H, arrow heads). Scale bars: A–F: 25 μm; G,H: 10 μm.
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throughout the neurosphere, whereas progeny remaining at the periphery continued to proliferate.

The spatial pattern of cell proliferation and its relationship to cell migration in ENS neurospheres is reminiscent of that in the CNS in which progenitor proliferation in germinal zones located peripherally is followed by the migration of postmitotic cell progeny to deeper layers of the CNS [25]. Furthermore, evidence has been presented from *in vivo* studies that extraganglionic post-mitotic ENS progenitor cells migrate into ganglia in the adult nervous system [26]. Thus, progenitor cell behavior in ENS neurospheres mimics that of both CNS and ENS progenitors *in vivo*. Future analysis of the relationship between proliferation and migration in neurospheres will help to establish the mechanisms coordinating this general behavior of neural progenitor cells.

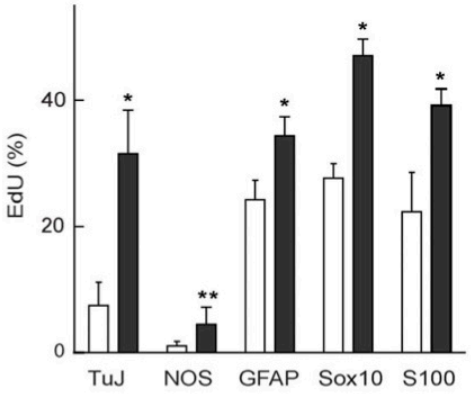

Figure 4. Analysis of proliferation of mouse neurosphere cells expressing neuronal and glial markers. Neurosphere cells were dissociated and allowed to attach to chamber slides immediately after a 1 h pulse of 10 μM EdU (open columns), or after a 96 h chase in the absence of EdU (closed columns). The vertical axis shows the percentage of cells positive for specific phenotypes that had also incorporated EdU. Error bars represent SEM (n = 3 separate experiments). A two-tailed t-test was performed for differences between before and after chase (open and closed columns) for each marker. * $p<0.05$; ** $p<0.075$.
doi:10.1371/journal.pone.0054809.g004

Our earlier studies provided preliminary evidence that the proportion and phenotypes of neurons in secondary and tertiary ENS neurospheres remain constant, implying that progenitor cell proliferation and differentiation of their progeny are closely linked [19]. We show here that very few cells that had incorporated the thymidine analog also expressed the neuronal markers Tuj1 or NOS immediately after the pulse. However, after a chase period of 96 h the numbers of dual-labeled cells had increased significantly, indicating that neuronal differentiation occurs after the neurosphere cells had withdrawn from the cell cycle. This conclusion is consistent with a detailed earlier study which clearly demonstrated a close relationship between the timing of withdrawal of ENS progenitor cells from the cell cycle and the differentiation of specific neuronal phenotypes *in vivo* [27]. Thus, similar control mechanisms may be responsible for the proliferative and differentiative behavior of ENS neural progenitor cells both *in vivo* and in neurospheres *in vitro*.

Injury to the postnatal ENS results in a mitotic response of cells which may either be glial in origin [28,29], or possibly be a small number of quiescent progenitor cells remaining in or close to the ENS ganglia [26]. These proliferating cells have the properties of multipotent neuronal and glial progenitor cells, although transplantation studies have shown that the environment *in vivo* can affect their differentiation by biasing it towards a glial phenotype [28]. It is of interest to note that while neuronal differentiation in neurospheres occurred following a delay after cell proliferation, a larger proportion of neurosphere cells expressing glial markers were labeled with the thymidine analog immediately after the short pulse. This reflects the previous observations indicating that neural crest-derived cells expressing glial markers are able to proliferate *in vitro* [28,29]. Indeed, it is now well established that GFAP-expressing cells are multipotent progenitors for both neurons and glia in the developing CNS [30]. In this context it should be noted that the procedure used to isolate ENS cells during the production of neurospheres constitutes an injury which

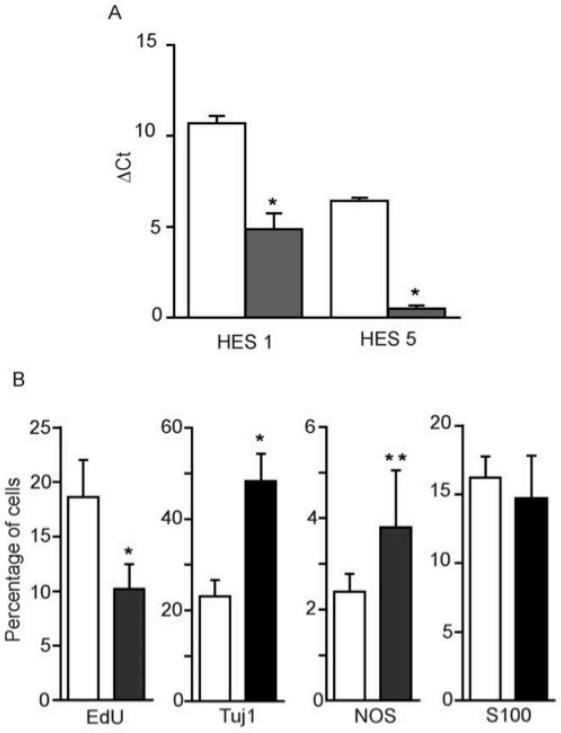

Figure 5. Effects of DAPT-mediated γ-secretase inhibition on cell proliferation and expression of neuronal and glial markers by mouse neurosphere cells. After 2 weeks culture mouse neurospheres were cultured for a further 96 h in the presence of 20 μM DAPT (shaded columns) or DMSO solvent control (clear columns). A: Levels of Hes1 and Hes5 mRNA determined by q-PCR. Columns show the ΔC_t values, normalised to β-actin levels (± SEM, means of 3 individual experiments). B: Expression of neuronal and glial markers. Cells dissociated from the neurospheres were allowed to adhere to Permanox slides before fixation and staining for EdU, Tuj1, NOS and S100. Nuclei were counter-stained with DAPI. Fluorescent cells were counted in 5 random optical fields in each chamber using a 40 x oil objective. Error bars are ± SEM, (values from 3–5 experiments). A two-tailed t-test was performed for differences between open and closed columns for each marker. *p<0.01.
doi:10.1371/journal.pone.0054809.g005

is likely to contribute to stimulation of cell proliferation in ENS neurospheres [28,29].

Notch receptors and their ligands are expressed in the ENS [17,31], and evidence for a role for Notch signaling during ENS development has been provided by showing that inhibition of the Notch pathway resulted in defective ENS development in embryonic mice, associated with premature neurogenesis and reduction in ENS progenitor cells [17]. We show here that blocking the canonical Notch signaling pathway in ENS neurosphere cells by either siRNA directed against RBPjκ or chemical inhibition with DAPT inhibits progenitor cell proliferation while increasing the numbers of cells expressing the neuronal marker Tuj1. Although both embryonic mouse and neonatal human ENS neurosphere cells display Notch-dependent proliferation and inhibition of neuronal differentiation, the Notch signaling in neurospheres did not result in a permanent shift in ENS progenitor cell potential from neurogenic to gliogenic, as has been reported for other neural crest cell derivatives [32].

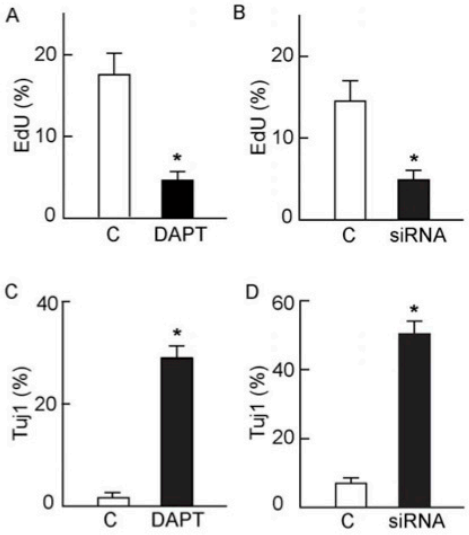

Figure 6. Effects of DAPT and RBPjκ siRNA-mediated Notch inhibition on human neurosphere cell proliferation and neuronal differentiation. A and C: Cells dissociated from human neurospheres were allowed to adhere to Permanox tissue culture slides and cultured with and without 20 μM DAPT. After 96 h the cells were fixed and stained for Tuj1, and nuclei counterstained with DAPI. Fluorescent cells were counted in 5 random optical fields in each chamber. Error bars are ± SEM, (values from 3–5 experiments). A: Significantly fewer DAPT-treated cells incorporated EdU than controls (* p<0.001 ANOVA) while expression of Tuj1 (C) was markedly increased over control levels (* p<0.001 ANOVA). B and D: The effect of siRNA knockdown on the human neurosphere cells prepared and cultured under the same conditions as in A and C. Relative to controls transfected with an irrelevant siRNA, after 96 h siRNA transfection with HsRBPJ_3 siRNA specific for RBPjκ had (B) a lower rate of proliferation (* p<0.001, ANOVA) and (D) increased expression of Tuj1 (* p<0.001, ANOVA). Error bars are ± SEM (n = 3).
doi:10.1371/journal.pone.0054809.g006

It remains to be established which other factors modulate the Notch-dependent maintenance of progenitor cell self-renewal *in vivo* and *in vitro*. In this context it is important to note that sonic hedgehog has recently been shown to increase Notch signaling in ENS progenitor cells via induction of the Notch ligand DLL3 [18]. Furthermore, evidence has been presented indicating that Notch signaling may be compromised in HSCR bowel [33]. Our in vitro work presented here provides the basis for future experiments to determine if Notch signaling regulates ENS progenitor cell behavior after transplantation, and if this can be manipulated to improve the outcome for HSCR patients.

Supporting Information

Figure S1 Neurosphere cell labeling during 4 day chase after a 1 h pulse of BrdU. Primary mouse neurospheres previously cultured in suspension for 15 days were labeled with a 1 h pulse of 10 μM BrdU. After BrdU removal and washing, an aliquot of the neurospheres was fixed and the remaining neurospheres were then cultured further, removing aliquots for fixation at 1, 2 and 4 days. BrdU immunostaining (red) was performed on 8 μm cryostat sections taken from the equatorial region of the neurospheres, followed by counterstaining of nuclei by DAPI (blue). Scale bar = 25 μm.
(TIF)

Figure S2 Double labeling of neurosphere cells for neural cell markers and EdU incorporation. Primary mouse neurospheres previously cultured for 15 days under non-adherent conditions were labeled with a 1 h pulse of 10 μM EdU. The neurospheres were then dissociated and allowed to attach after which they were fixed and permeabilized before immunostaining for the neural cell markers shown and processing to reveal EdU incorporation. The montages shown were constructed in Adobe Photoshop from 3 separate images captured to demonstrate the EdU incorporation, immunofluorescence and phase contrast images. Scale bars = 25 μm.
(TIF)

Figure S3 Agarose gel electrophoresis of qPCR products after DAPT treatment of neurospheres. The PCR products obtained from the experiment in Fig. 5A was electrophoresed in 2% agarose gels. Calibration standards (bp) are shown on the left hand side of each gel. PCR product sizes were: Hes1 = 354 bp, Hes5 = 183 bp and 269 bp and β-actin = 143 bp. The DNA in each excised band was sequenced to confirm PCR product identity; the double bands for Hes5 represent two splice variants amplified by the primer pair used.
(TIF)

Figure S4 Confirmation of RBPjκ knockdown in human neurospheres. Mature 2nd to 3rd passage human neurospheres were dissociated and cultured on fibronectin coated chamber slides for 96 h. The dissociated cells were transfected with HsRBPJ_3 siRNA knockdown specific for RBPjκ or a corresponding negative control. Levels of RBPjκ were determined by qPCR. Columns show the normalised ΔC_t values (± SEM, n = 3). * = $P<0.01$.
(TIF)

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Author Contributions

Obtained ethical permission for acquisition of human tissue: DJW SEK. Conceived and designed the experiments: ST DJW BW SEK DE. Performed the experiments: ST DJW SD. Analyzed the data: ST DJW SD BW DE. Contributed reagents/materials/analysis tools: ST DJW BW DE. Wrote the paper: ST DJW BW DE.

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